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(57) Abstract

The invention is directed in part towards methods of treating urinary incontinence using gene therapy techniques. The methods provide for the delivery and expression of growth factors or neurotrophic factors in mammalian tissues.

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DESCRIPTION

TREATMENT FOR URINARY INCONTINENCE USING GENE THERAPY TECHNIQUES

BACKGROUND

The invention relates in part to a treatment of urinary incontinence. Urinary incontinence (UI) is defined as the "involuntary loss of urine which is a social or hygienic problem and objectively demonstrable." Abrams et al., 1990, *J. Obstet. Gynecol. 97*: 1-16. It has been estimated that as many as 5% of men and 25% of woman between the ages of 15 and 64 years of age are affected by UI. Johnson and Gary, 1995, *J. Wound Ostomy Continence Nursing 22*: 8-16. UI has been reported as one of the leading causes of nursing home admissions, with 50% of nursing home residents having some degree of UI. The economic repercussions of UI, based on a mid-1980s estimate, are reported in the amounts of $7 billion in the community and $3.3 billion in nursing homes annually. Wyman et al., 1987, *Obstet. Gynecol. 70*: 378.

Proper urinary function of continent individuals depends in part upon the coordination between the urethral sphincter and its innervating neurons in the peripheral nervous system. The urethral sphincter, which is relaxed when the bladder is empty, contracts as the bladder fills with urine. Once the bladder accumulates approximately 150 mL of urine, the urethral sphincter of continent individuals relaxes in conjunction with pelvic floor muscles, which is achieved by the integrated network of neurons between the two

muscle groups. Relaxation of these muscles voids the bladder.

Thus, a urethral sphincter characterized by a lack of muscle tone can result in a constant leakage of urine, known as stress UI. Furthermore, decreased neuronal innervation to the urethral sphincter can cause unpredictable urinary leakage due to a lack of sensory signals linking bladder volume to sphincter relaxation. The importance of pelvic floor muscular integrity with regards to continence is underscored by the correlation between childbirth induced damage to this muscle group and UI. DeLancey, 1993, *New England J. Medicine*: 1956-1957.

The economic drain that UI imposes upon the community as well as nursing homes has created a great need for treatments of the disorder. Treatments currently used to treat UI include behavioral, pharmacologic, and surgical procedures. Although some surgical procedures successfully treat urethral obstruction, none abolish over active micturition or restore normal micturition. Blavias et al., 1996, *J. Endourology 10*: 213-216. These surgical procedures can also give rise to complications. Neale, 1995, *Curr. Opin. Obstetrics Gynecology* 7: 400-403.

Pharmacological treatments of UI are limited to systemic drugs and injection of periurethral bulking reagents, such as lactone-based polymers (EP 711764 A1), collagen, and polytetrafluoroethylene. Systemic drugs include smooth muscle contractors such as diamino cyclobutene-3,4-diones (US 5,530,025), anticholinergic and antispasmodic drugs such as oxybutanin (WO 96/23492) and propantheline, α-sympathomimetics such as

phenylpropanolamine and ephedrine, calcium channel blockers such as verapamil and nifedipine, hormone treatments with orally administered estradiol, and tricyclic antidepressants such as imipramine. Although some of these drugs indirectly increase the tone of muscles surrounding the urinary tract, the drugs are not known to increase neuronal innervation to these muscles. The systemic nature of these drugs can also cause multiple side effects.

SUMMARY OF THE INVENTION

The invention relates to methods of treating urinary incontinence (UI) using gene therapy techniques. The economic drain that UI imposes upon the community as well as nursing homes has developed a great medical need for treatments of the disorder.

Treatments currently used to treat UI are either invasive surgical procedures that only treat urethral obstruction, or non-specific pharmacological therapies. Gene therapy treatment is a revolutionary approach towards treating UI as the treatment is tissue specific. The specificity of the gene therapy treatment lies in the fact that a gene product can be targeted to a tissue associated with UI by direct delivery of a nucleic acid vector that limits the expression of a gene product within a tissue. Gene therapy techniques also provide versatility as they can utilize a multitude of genes, including those that enhance muscle tone and neuronal innervation to the muscle or muscles associated with UI. One such gene is IGF-1, which increases muscle tone and strength as well as neuronal innervation to the muscle tissue expressing the hormone.

Thus in a first aspect, the invention features a method of treating urinary incontinence in mammals. The method comprises the step of delivering a nucleic acid vector for the expression of a growth factor or neurotrophic factor in a tissue or tissues.

The term "urinary incontinence" refers to a medical condition in which a patient involuntarily loses urine. The condition is also defined as a social or hygienic problem which is objectively demonstrable.

The term "treating" as used herein refers to at least partially restoring urinary continence to a urinary incompetent individual. "Treating" refers to improving the control of urine flow and decreasing the involuntary loss of urine.

The term "mammals" as used herein refers to any vertebrate that reproduces by live birth following an internal gestation period. Examples of mammals are preferably cats, dogs, rabbits, and pigs and most preferably humans.

The term "vector" as used herein refers to a nucleic acid, e.g., DNA derived from a plasmid, cosmid, phasmid or bacteriophage or synthesized by chemical or enzymatic means, into which one or more fragments of nucleic acid may be inserted or cloned which encode for particular genes. The vector can contain one or more unique restriction sites for this purpose, and may be capable of autonomous replication in a defined host or organism such that the cloned sequence is reproduced. The vector may have a linear, circular, or supercoiled configuration and may be complexed with other vectors or other materials for certain purposes. The components of a vector can include but are not limited to a DNA

molecule incorporating: (1) a sequence encoding a therapeutic or desired product; and (2) regulatory elements for transcription, translation, RNA stability and replication.

The vector can be used to provide expression of a nucleic acid sequence in tissue. In the present invention this expression is enhanced by providing stability to an mRNA transcript from the nucleic acid sequence and/or secretion of the therapeutic protein. Expression includes the efficient transcription of an inserted gene or nucleic acid sequence within the vector. Expression products may be proteins including but not limited to pure protein (polypeptide), glycoprotein, lipoprotein, phosphoprotein, or nucleoprotein. Expression products may also be RNA. The nucleic acid sequence is contained in a nucleic acid cassette. Expression of the nucleic acid can be continuous or controlled by endogenous or exogenous stimuli.

The term "control" or "controlled" as used herein relates to the expression of gene products (protein or RNA) at sufficiently high levels such that a therapeutic effect is obtained. Levels that are sufficient for therapeutic effect are lower than the toxic levels. Levels of expression for therapeutic effect within selected tissues corresponds to reproducible kinetics of uptake, elimination from cell, post-translational processing, and levels of gene expression, and, in certain instances, regulated expression in response to certain endogenous or exogenous stimuli (e.g., hormones, drugs).

The term “growth factor” as used herein generally refers to a polypeptide that binds to a specific receptor on the outer surface of a cell. The general effects of growth factors are cell growth, cell proliferation, and cell differentiation. Examples of growth factors are epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and insulin-like growth factor (IGF-1 and IGF-II).

The term “neurotrophic factor” as used herein generally refers to a polypeptide that, upon binding a specific type of receptor on the cell surface, causes survival, stimulation, grwoth, or proliferation of neuronal tissue. Examples of neurotrophic factors are nerve growth factor (NGF), interleukins (IL-15), brain-derived neurotrophic factor (BDNF), neurotrophins (NT-3, NT-4/5, NT-6), cilliary neurotrophic factor (CNTF), glial-derived growth factor (GDNF), and leukaemic inhibitory factor (LIF).

The term “delivering” refers to a method of transferring a vector from a medical device to a tissue or tissues. The method can be accomplished using a hypodermic needle attached to a syringe, a method well known to those skilled in the art.

The term "tissue" as used herein refers to a collection of cells specialized to perform a particular function or can include a single cell. The cells may be of the same type or of different types.

In preferred embodiments, the invention relates to the method of treating urinary incontinence where the vector is contained within a formulation comprising a

solution having between 0.5% and 50% polyvinyl pyrrolidone (PVP).

The components of the formulation can, for example, act to stabilize the vector or to enhance transfection efficiency, but can also provide other functions. Preferably, the PVP has an average molecular weight of about 50,000 g/mol. Further information is disclosed in PCT US95/17038. However, another example of a formulation includes the vector with a cationic lipid (e.g., as described in U.S. Patent 4,897,355, issued January 30, 1990), and can also include a co-lipid, such as a neutral co-lipid.

In a preferred embodiment, the formulation includes about 5% PVP.

In reference to the formulations of this invention, the term "about" indicates that in preferred embodiments, the actual value for a particular parameter is in the range of 50%-200% of the stated value.

In another preferred embodiment, the invention relates to the method of treating urinary incontinence, where the tissue is myogenic. Because the urinary system is comprised mostly of smooth muscle, the invention preferably relates to tissues that are smooth muscle.

The term "myogenic" refers to muscle tissue or cells. The muscle tissue or cells can be *in vivo, in vitro,* or *in vitro* tissue culture and capable of differentiating into muscle tissue. Myogenic cells include skeletal, heart and smooth muscle cells. Genes are termed "myogenic" or "myogenic-specific" if they are expressed or expressed preferentially in myogenic cells. Vectors are termed "myogenic" or "myogenic-specific" if

they function preferentially in myogenic muscle tissue or cells. Myogenic activity of vectors can be determined by transfection of these vectors into myogenic cells in culture, injected into intact muscle tissue, or injected into mammalian oocytes to be stably incorporated into the genome to generate transgenic animals which express the protein or RNA of interest in myogenic cells.

The term "non-myogenic" refers to tissue or cells other than muscle. The tissues or cells can be *in vivo, in vitro,* or *in vitro* tissue culture.

In another preferred embodiment, the invention relates to the method of treating urinary incontinence, where the myogenic tissue is selected from the group consisting of urethral sphincter musculature, detrusor musculature, and pelvic floor musculature.

The terms "urethral sphincter musculature", "detrusor musculature", and "pelvic floor musculature" refer to the myogenic tissue that comprise the urinary system in mammals. For example, the urethral sphincter is a muscular valve that resists the flow of urine when contracted. Detrusor musculature surrounds the bladder and contracts when the bladder fills with urine. It is the contraction of the detrusor musculature that sends the signal to a mammal's brain that the bladder is filling. Urination in the continent individual begins when the urethral sphincter, detrusor musculature, and pelvic floor musculature relax.

Another preferred embodiment of the invention relates to the method of treating urinary incontinence, where the delivery is accomplished by injecting the vector using a hypodermic needle or hypospray apparatus.

The gene therapeutic agent contained within a formulation can be injected into a specific tissue using a hypodermic needle. This type of technique is routinely practiced by persons skilled in the art.

In a preferred embodiment, the invention relates to the method of treating urinary incontinence, where the vector comprises: (a) a nucleic acid cassette containing a nucleotide sequence encoding a gene; (b) a 5' flanking region including one or more sequences necessary for expression of the nucleic acid cassette, where the sequences include a promoter element selected from the group consisting of skeletal muscle α-actin gene promoter, smooth muscle γ-actin gene promoter, and cytomegalovirus promoter; (c) a linker connecting the 5' flanking region to a nucleic acid, where the linker has a position for inserting the nucleic acid cassette, and where the linker lacks the coding sequence of a gene with which it is naturally associated; and (d) a 3' flanking region, including a 3'-UTR or a 3'NCR or both, where the 3' flanking region is 3' to the position for inserting the nucleic acid cassette, and where the 3' flanking region comprises a sequence from a growth hormone 3'-UTR.

The term "flanking region" as used herein refers to nucleotide sequences on either side of an associated gene. Flanking regions can be either 3' or 5' to a particular gene in question. In general, flanking sequences contain elements necessary for regulation of expression of a particular gene. Such elements include, but are not limited to, sequences necessary for efficient expression, as well as tissue specific expression. Examples of sequences necessary for

efficient expression can include specific regulatory sequences or elements adjacent to or within the protein coding regions of DNA. These elements, located adjacent to the gene, are termed cis-acting elements. The signals are recognized by other diffusible biomolecules in *trans* to alter the transcriptional activity. These biomolecules are termed trans-acting factors. *Trans*-acting factors and *cis*-acting elements have been shown to contribute to the timing and developmental expression pattern of a gene. *Cis*-acting elements are usually thought of as those that regulate transcription and are usually found within promoter regions and within upstream (5') or downstream (3') DNA flanking regions.

Flanking DNA with regulatory elements that regulate expression of genes in tissue may also include modulatory or regulatory sequences which are regulated by specific factors, such as glucocorticoids, androgens, progestins, antiprogestins (PCT US93/04399; PCT US96/04324), vitamin D_3 and its metabolites, vitamin A and its metabolites, retinoic acid, calcium as well as others.

"Modulatory" or "regulatory" sequences as used herein refer to sequences which may be in the 3' or 5' flanking region, where such sequences can enhance activation and/or suppression of the transcription of the associated gene.

"Responsive" or "respond" as used herein refers to the enhancement of activation and/or suppression of gene transcription as discussed below.

"Metabolite" as used herein refers to any product from the metabolism of the regulatory factors which regulate gene expression.

In addition to the above, either or both of the 3' or 5' flanking regions can cause tissue specificity. Such tissue specificity provides expression predominantly in a specified cell or tissue.

"Predominantly" as used herein means that the gene associated with the 3' or 5' flanking region is expressed to a higher degree only in the specific tissue as compared to low expression or lack of expression in nonspecific tissue. The 3' or 5' flanking regions singularly or together as used herein can provide expression of the associated gene in other tissues but to a lower degree than expression in tissues or cells where expression is predominate. Expression is preferentially in the specified tissue. Such predominant expression can be compared with the same magnitude of difference as will be found in the natural expression of the gene (i.e. as found in a cell *in vivo*) in that particular tissue or cell type as compared with other nonspecific tissues or cells. Such differences can be observed by analysis of mRNA levels or expression of natural gene products, recombinant gene products, or reporter genes. Furthermore, northern analysis, X gal immunofluorescence or CAT assays as discussed herein and as known in the art can be used to detect such differences.

The 3' flanking region contains sequences or regions, e.g. 3'UTR and/or 3' NCR, which regulate expression of a nucleic acid sequence with which it is associated. The 3' flanking regions can provide tissue-specific expression to an associated gene. The 3' flanking region also contains a transcriptional termination signal.

The term "3' flanking region" as used herein includes that portion of a naturally occurring sequence 3' to the transcribed portion of the gene which are responsible for mRNA processing and/or tissue-specific expression. That portion can be readily defined by known procedures. For example, the portions of a 3' flanking region which are responsible for mRNA stability and/or tissue-specific expression can be mapped by mutational analysis or various clones created to define the desired 3' flanking region activity in a selected vector system.

The 3' flanking region can contain a 3'UTR and/or a 3' NCR. The term "3' untranslated region" or "3'UTR" refers to the sequence at the 3' end of structural gene which is transcribed from the DNA but not translated into protein. This 3'UTR region does not contain a poly(A) sequence, but generally contains a site at which a poly(A) sequence is added. Poly (A) sequences are only added after the transcriptional process.

Myogenic-specific 3'UTR sequences can be used to allow for specific stability in muscle cells or other tissues. As described below, myogenic-specific sequences refers to gene sequences normally expressed in muscle cells, e.g., skeletal, heart and smooth muscle cells. Myogenic specific mRNA stability provides an increase in mRNA stability within myogenic cells. The increase in stability provides increased expression. The 3'UTR and 3' NCR sequences singularly or together can provide a higher level of mRNA accumulation through increased mRNA stability. Thus, increased expression and/or stability of mRNA leads to increased levels of protein production.

The term "3' non-coding region" or "3'NCR" is a region which is adjacent to the 3'UTR region of a structural gene. The 3'NCR region generally contains a transcription termination signal. Once transcription occurs and prior to translation, the RNA sequence encoded by the 3'NCR is usually removed so that the poly(A) sequence can be added to the mRNA. The 3'NCR sequences can also be used to allow mRNA stability as described above. The 3'NCR may also increase the transcription rate of the nucleic acid cassette.

Either or both of the 3'UTR and 3' NCR sequences can be selected from a group of myogenic-specific genes. Examples of myogenic-specific genes include the skeletal α-actin gene, fast myosin-light chain 1/3 gene, myosin-heavy chain gene, troponin T gene, acetylcholine receptor subunit genes and muscle creatinine kinase gene.

In reference to 3' flanking regions of this invention, the term "growth hormone" refers to a gene product identified as a growth hormone, for example, human growth hormone or bovine growth hormone. Homologous gene sequences are known in the art for a variety of different vertebrate animals. In different embodiments, the vectors can incorporate 3' sequences, including 3' UTR sequences from such growth hormone genes. The 3' sequence can be modified from the sequence naturally found in the animal, for example by the deletion of ALU repeat sequence from human growth hormone 3' UTR. The deletion of ALU repeats or ALU repeat-like sequences can be performed with a variety of 3' sequences; such deletion generally reduces the rate of homologous recombination. A variety of other

modifications may also be made without destroying the tissue targeting, stabilizing, and secretion properties of the 3' sequence.

The 5' flanking region is located 5' to the associated gene or nucleic acid sequence to be expressed. Just as with the 3' flanking region, the 5' flanking region can be defined by known procedures. For example, the active portion of the 5' flanking region can be mapped by mutational analysis or various clones of the 5' flanking region created to define the desired activity in a selected vector. The 5' flanking region can include, in addition to the above regulatory sequences or elements, a promoter, a TATA box, and a CAP site, which are in an appropriate relationship sequentially and positionally for the expression of an associated gene.

In this invention, "sequences necessary for expression" are those elements of the 5' flanking region which are sequentially and positionally in an appropriate relationship to cause controlled expression of a gene within a nucleic acid cassette. Expression is controlled to certain levels within tissues such that the expressed gene is useful for gene therapy and other applications involving gene delivery. The 5' sequence can contain elements which regulate tissue-specific expression or can include portions of a naturally occurring 5' element responsible for tissue-specific expression.

The term "promoter," as used herein refers to a recognition site on a strand of DNA to which RNA polymerase binds. The promoter usually is a DNA fragment of about 100 to about 200 base pairs (in

eukaryotic genes) in the 5' flanking DNA upstream of the CAP site or the transcriptional initiation start site. The promoter forms an "initiation complex" with RNA polymerase to initiate and drive transcriptional activity. The complex can be modified by activating sequences termed "enhancers" or inhibitory sequences termed "silencers". The promoter can be one which is naturally (i.e., associated as if it were within a cell *in vivo*) or non-naturally associated with a 5' flanking region.

A variety of promoters can be used. Some examples include thymidine kinase promoter, myogenic-specific promoters including skeletal α-actin gene promoter, fast myosin light chain 1 promoter, myosin heavy chain promoter, troponin T promoter, and muscle creatinine kinase promoter, as well as non-specific promoters including the cytomegalovirus immediate early promoter and the Rous Sarcoma virus LTR. These promoters or other promoters used with the present invention can be mutated in order to increase expression of the associated gene. Furthermore a promoter may be used by itself or in combination with elements from other promoters, as well as various enhancers, transcript stabilizers, or other sequences capable of enhancing function of the vector.

"Mutation" as used herein refers to a change in the sequence of genetic material from normal causing a change in the functional characteristics of the gene. This includes gene mutations where only a single base is changed in the natural gene promoter sequences or multiple bases are changed.

The term "intron" as used herein refers to a section of DNA occurring in a transcribed portion of a gene which is included in a precursor RNA but is then excised during processing of the transcribed RNA before translation occurs. Intron sequences are therefore not found in mRNA nor translated into protein. The term "exon" as used herein refers to a portion of a gene that is included in a transcript of a gene and survives processing of the RNA in the cell to become part of a mature mRNA. Exons generally encode three distinct functional regions of the RNA transcript. The first, located at the 5' end which is not translated into protein, termed the 5' untranslated region (5' UTR), signals the beginning of RNA transcription and contains sequences that direct the mRNA to the ribosomes and cause the mRNA to be bound by ribosomes so that protein synthesis can occur. The second contains the information that can be translated into the amino acid sequence of the protein or function as a bioactive RNA molecule. The third, located at the 3' end is not translated into protein, i.e. 3' UTR, contains the signals for termination of translation and for the addition of a polyadenylation tail (poly(A). In particular, the 3' UTR as defined above can provide mRNA stability. The intron/exon boundary will be that portion in a particular gene where an intron section connects to an exon section. The terms "TATA box" and "CAP site" are used as they are recognized in the art.

The term "linker" as used herein refers to DNA which contains the recognition site for a specific restriction endonuclease. Linkers may be ligated to the ends of DNA fragments prepared by cleavage with some

other enzyme. In particular, the linker provides a recognition site for inserting the nucleic acid cassette which contains a specific nucleic sequence to be expressed. This recognition site may be but is not limited to an endonuclease site in the linker, such as Cla-I, Not-I, Xmal, Bgl-II, Pac-I, Xhol, Nhel, Sfi-I. A linker can be designed so that the unique restriction endonuclease site contains a start codon (e.g. AUG) or stop codon (e.g. TAA, TGA, TCA) and these critical codons are reconstituted when a sequence encoding a protein is ligated into the linker. Such linkers commonly include an NcoI or SphI site.

The term "leader" as used herein refers to a DNA sequence at the 5' end of a structural gene which is transcribed and translated along with the gene. The leader usually results in the protein having an n-terminal peptide extension sometimes called a pro-sequence. For proteins destined for either secretion to the extracellular medium or the membrane, this signal sequence directs the protein into endoplasmic reticulum from which it is discharged to the appropriate destination. The leader sequence normally is encoded by the desired nucleic acid, synthetically derived or isolated from a different gene sequence. A variety of leader sequences from different proteins can be used in the vectors of the present invention. Some non-limiting examples include gelsolin, albumin, fibrinogen and other secreted serum proteins.

The term "nucleic acid cassette" as used herein refers to the genetic material of interest which codes for a protein or RNA. The nucleic acid cassette is positionally and sequentially oriented within the vector

such that the nucleic acid in the cassette can be transcribed into RNA, and when necessary, translated into a protein in the transformed tissue or cell. Preferably, the cassette has 3' and 5' ends adapted for ready insertion into a vector, *e.g.*, it has restriction endonuclease sites at each end. In the vectors of this invention, a nucleic acid cassette contains a sequence coding for insulin-like growth factor I (IGF-I), *e.g.*, human IGF-I.

The term "gene", e.g., "myogenic genes," as used herein refers to those genes exemplified herein and their equivalence in other animal species or other tissues. Homologous sequences (i.e. sequences having a common evolutionary origin representing members of the same superfamily) or analogous sequences (i.e. sequences having common properties though a distinct evolutionary origin) are also included so long as they provide equivalent properties to those described herein. It is important in this invention that the chosen sequence provide the enhanced levels of expression, expression of the appropriate product, and/or tissue-specific expression as noted herein. Those in the art will recognize that the minimum sequences required for such functions are encompassed by the above definition. These minimum sequences are readily determined by standard techniques exemplified herein.

Another preferred embodiment relates to the method of the invention where the growth factor or neurotrophic factor is selected from the group consisting of PDGF, EGF, FGF, NGF, BDNF, IL-15, NT-3, NT-4/5, NT-6, CNTF, LIF, and GDNF.

In yet another preferred embodiment, the invention relates to the method of treating urinary incontinence, where the growth factor is IGF-1 or IGF-II.

In another preferred embodiment, the invention relates to the method of treating urinary incontinence, where the IGF-1 gene is isolated from a human organism. Methods of isolating a gene of known sequence from nearly any organism are well known to those skilled in the art of nucleic acid cloning techniques.

In a yet another preferred embodiment, the invention relates to the method of treating urinary incontinence, where the human IGF-I gene is a synthetic sequence, which differs from a natural human IGF-I coding sequence. It is preferred that the sequence utilize optimal codon usage; preferably at least 50%, 70%, or 90% of the codons are optimized. Thus, in preferred embodiments the synthetic DNA sequence has at least 80, 90, 95, or 99% sequence identity to the sequence of SEQ ID NO. 1. In a particular preferred embodiment, the synthetic DNA sequence has at least 95% identity, more preferably at least 99% identity, and most preferably 100% identity to the sequence of SEQ ID NO. 4.

In another preferred embodiment, the nucleotide sequence encoding human IGF-I has the sequence designated by SEQ ID NO. 4, included herein.

In a preferred embodiment, the invention relates to a method of treating urinary incontinence, where the promoter from the skeletal muscle α-actin gene or the smooth muscle γ-actin gene is isolated from a chicken. Specifically, this can include a promoter sequence which may be linked with other 5' UTR sequences, which can

include an intron. While vectors using the chicken skeletal α-actin promoter and/or other 5' flanking sequences are exemplified herein, the 5' sequences for α-actin genes are highly conserved, therefore, such 5' α-actin sequences can be utilized from other vertebrate species, including, for example, human.

In another preferred embodiment, the invention relates to the method of treating urinary incontinence, where the promoter from the skeletal α-actin gene or the smooth muscle γ-actin gene is isolated from a human.

In yet another preferred embodiment the growth hormone 3'-UTR is from a human growth hormone gene. The growth hormone preferably includes a poly(A) signal. This sequence can be linked immediately following the natural translation termination codon for a cDNA sequence coding for the protein or RNA to be expressed. As discussed above, these regions can be further and more precisely defined by routine methodology, *e.g.*, deletion or mutation analysis or their equivalents.

The 5' or 3' sequences may have a sequence identical to the sequence as naturally found, but may also have changed sequences which provide equivalent function to a vector in which such 5' or 3' sequences are incorporated. Such a change, for example, could be a change of ten nucleotides in any of the above regions. In particular, such changes can include the deletion of ALU repeat sequences from the 3' UTR. This is only an example and is non-limiting.

In addition, an embodiment of the vector may contain a regulatory system for regulating expression of the nucleic acid cassette. The term "regulatory system" as used herein refers to cis-acting or trans-acting

sequences incorporated into the above vectors which regulate in some characteristic the expression of the nucleic acid of interest as well as trans-acting gene products which are co-expressed in the cell with the above described vector. Regulatory systems can be used for up-regulation or down regulation of expression from the normal levels of expression or existing levels of expression at the time of regulation. The system contributes to the timing and developmental expression pattern of the nucleic acid.

One construction of a regulatory system includes a chimeric *trans*-acting regulatory factor incorporating elements of a serum response factor capable of regulating expression of the vector in a cell. The chimeric transacting regulatory factor is constructed by replacing the normal DNA binding domain sequence of the serum response factor with a DNA binding domain sequence of a receptor. The serum response factor has a transactivation domain which is unchanged. The transactivation domain is capable of activating transcription when an agent or ligand specific to the receptor binding site binds to the receptor. Thus, regulation can be controlled by controlling the amount of the agent.

The DNA binding domain sequence of a receptor, incorporated into the chimeric trans-activating regulatory factor, can be selected from a variety of receptor groups including but not limited to vitamin, steroid, thyroid, orphan hormone, retinoic acid, thyroxine, or GAL4 receptors. The chimeric trans-activating regulator factor is usually located within the sequence of the promoter. In one preferred

embodiment the promoter used in the vector is the α-actin promoter and the receptor is the vitamin D receptor.

"Receptor" as used herein includes natural receptors (i.e., as found in a cell *in vivo*) as well as anything that binds alike and causes compartmentalization changes in a cell.

Another embodiment of the regulatory system includes the construction of a vector with two functional units. One functional unit expresses a receptor. This functional unit contains elements required for expression including a promoter, a nucleic acid sequence coding for the receptor, and a 3' UTR and/or a 3' NCR. The second functional unit expresses a therapeutic protein or RNA and contains, in addition, a response element corresponding to the receptor, a promoter, a nucleic acid cassette, and a 3' UTR and/or a 3' NCR. These functional units can be in the same or separate vectors.

The first functional unit expresses the receptor. It is favorable to use a receptor not found in high levels in the target tissue. The receptor forms an interaction, e.g., ionic, non-ionic, hydrophobic, H-bonding, with the response element on the second functional unit prior to, concurrent with, or after the binding of the agent or ligand to the receptor. This interaction allows the regulation of the nucleic acid cassette expression. The receptor can be from the same nonlimiting group as disclosed above. Furthermore, the vector can be myogenic specific by using myogenic specific 3' UTR and/or 3' NCR sequences.

In an exemplary preferred embodiment the plasmid can be pIG0552 or a plasmid comprising a nucleotide sequence which is the same as the sequence of pIG0552. This is only an example and is meant to be non-limiting. Thus, sequence changes or variations can be made to one or more of the sequence elements, such as the 5' and 3' flanking regions. The sequences utilized for this exemplary vector have the advantage of providing an IGF-I RNA splice product which produces a polypeptide having a signal sequence of equal length as a form found naturally in muscle and many other tissues.

In this context, the word "same" means that the sequences are functionally equivalent and have a high degree of sequence identity. However, the sequences may have a low level of sequence differences, such as by substitution, deletion, or addition of one or more nucleotides. Such sequences will preferably be less than 10%, more preferably less than 5%, and still more preferably less than 1% of the total sequence.

In particular embodiments, the vectors of the above aspect may alternatively comprise, consist essentially of, or consist of the stated elements or sequences.

By "comprising" it is meant including, but not limited to, whatever follows the word "comprising". Thus, use of the term "comprising" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present. By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of". Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present. By "consisting essen-

tially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

In preferred embodiments, the vector may have the ALU repeat or ALU repeat-like sequence deleted from the 3'-UTR. An nucleic acid element can be readily deleted from a vector using nucleic acid recombinant techniques routinely utilized by those skilled in the art. An example of such a manipulation is described herein by example.

In additional preferred embodiments, the invention relates to methods of treating urinary incontinence utilizing the vector where the IGF-I gene is human IGF-I, the promoter from a skeletal muscle α-actin gene or smooth muscle γ-actin gene is from a chicken, and the growth hormone 3'-UTR is from a human growth hormone gene.

In preferred embodiments, the vector may comprise a nucleotide sequence where the 5' flanking region or the 3' flanking region or both regulates expression of the nucleic acid cassette predominately in a specific tissue or tissues.

In another preferred embodiment, the vector may comprise a nucleotide sequence where the 5' flanking region includes a promoter, a TATA box, a Cap site and a first intron and intron/exon boundary in an appropriate

relationship for expression of the nucleic acid cassette.

The terms "TATA box" and "Cap site" refer to nucleic acid sequences that facilitate the binding of RNA polymerase for transcription of gene therapeutic into a message strand of ribonucleic acid. The strand of ribonucleic acid can then be translated and expressed into protein in the targeted tissue. These terms are readily known to those skilled in the art, the nucleic acid elements are readily available to those skilled in the art, and the methods of manipulating these nucleic acid elements are routinely utilized by those skilled in the art. For a further description of these terms and methods of manipulating the TATA box, Cap site, intron and exon, and promoter nucleic acid elements *see* Sambrook, Fritsch, and Maniatis, 1989, *Molecular Cloning*, Cold Spring Harbor Laboratory Press, United States of America.

In yet another preferred embodiment, the vector may comprise a nucleotide sequence where the 5' flanking region further comprises a 5' mRNA leader sequence inserted between the promoter and the nucleic acid cassette.

In other preferred embodiments, the vector may comprise a nucleotide sequence where the vector further comprises an intron/5' UTR from a chicken skeletal α-actin gene.

In another preferred embodiment, the vector may comprise a nucleotide sequence where the vector further comprises an antibiotic resistance gene.

The term "antibiotic resistance gene" as used herein refers to a gene that produces an enzyme that transforms

an antibiotic into a compound which is non-toxic to the organism harboring the gene. Examples of such genes are described herein.

In yet another preferred embodiment, the vector may comprise a nucleotide sequence where the vector comprises a nucleotide sequence which is the same as the nucleotide sequence of plasmid pIG0552.

Other features and advantages of the invention will be apparent from the following detailed description of the invention in conjunction with the accompanying claims.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The methods of the invention are directed in part towards treating urinary incontinence using gene therapy techniques. The vectors and methods provide for the delivery and expression of growth factors or neurotrophic factors in mammalian cells, e.g., in human cells.

Growth factors have been shown to stimulate cell growth and proliferation and neurotrophic factors perform these functions specifically in neural tissue. For example, NGF regulates functionally important features, such as transmitter synthesis, of neurons during post natal life of organisms. Thoenen and Bard, 1980, *Pysiological Reviews 60*: 1284-1335. In contrast to NGF and its family members BDNF, NT-3, NT-4/5, and CTNF, FGF binds to a glycosaminoglycan heparin in order to bind its specific family of receptors. (Gaviol and Yayon, 1992, *FASEB Journal 6*: 3362-3369. FGFs have been shown to be survival factors for tissues and in particular ciliary and motoneurons. Unsiker et al.,

1992, *Curr. Opin. Neurobiology 1*: 671-678. EGF and PDGF are mitogenic growth factors involved growth and proliferation of cells. Neurotrophic factors in particular are being utilized for the treatment of amyotrophic lateral sclerosis (ALS) in clinical trials. Due to the growth and stimulatory effects of growth factors and neurotrophic factors, introducing these factors to degenerated muscles in the urinary system can improve UI by enhancing both their integrity and neural innervation.

In particular, it has been shown that insulin like growth factor (IGF-I) plays an important role in normal muscle development, muscle growth and hypertrophy, muscle regeneration and maintenance/regeneration of peripheral nerves. IGF-I and IGF-II are low molecular weight polypeptide hormones that stimulate growth and differentiation of many cell types, including myoblasts, nerve cells, fibroblasts, chondrocytes, osteoblasts, endothelial cells, and keratinocytes (Daughaday & Rotwein, 1989, *Endocrine Reviews* 10:68-91). IGF-I has a primary role in promoting the differentiation and growth of skeletal muscle. IGFs are key myogenic progression factors which propel myoblast cell division and fusion as well as stimulate late stage muscle growth and hypertrophy. Studies also indicate that myogenesis is stimulated by IGF stimulation of cells. Florini & Magri, 1989, Am. J. Physiol. (Cell Physiol.) 256:C701-C711. During the onset of fusion, the biosynthesis and secretion of IGFI/II and IGF binding proteins is naturally increased in myoblasts (Tollefsen et al., 1989, *J. Biol. Chem.* 264:13810-13817). This coincides with the appearance of muscle-specific gene products.

Direct evidence that IGF-I plays a role in muscle development was found when the single copy murine IGF-I gene was inactivated by homologous recombination (Powell-Braxton et al., 1993, *Genes Dev.* 7:2609-2617; Liu et al., 1993, *Cell* 75:59-72). By knocking out the IGF-I gene, severe muscle dystrophy and highly reduced myofibrillar organization of the skeletal muscle of these IGF-I mutants resulted. The majority of the mice died at birth due to respiratory failure, which was probably due to incomplete maturation of the diaphragm and intercostal muscles. These observations suggest that IGF-I is a central trophic growth factor required for embryonic muscle development and growth.

Recent studies also demonstrate a role for IGF-I in post-natal muscle growth and hypertrophy. Inclusion of IGF-I in the maintenance media of primary cultures of avian myofibers has been shown to elicit larger fiber diameters, a near doubling in myosin content and increases in protein stability and synthesis compared to untreated cultures (Vandenburg et al., 1991, Am. J. Physiol. 260:C475-C484). Administration of growth hormone to hypophysectomized rats resulted in a significant increase in IGF-I mRNA and a 50% increase in the mass of certain muscles (Englemann et al., 1989, *Mol. Cell. Endocrin.* 63:1-14). When the expression of IGF-I genes was increased through passive mechanical stretch and acute exercise, a corresponding increase in muscle hypertrophy was seen (Elgin et al., 1987, *Proc. Nat. Acad. Sci. USA* 84:3254-3258).

Studies also suggest that IGF-I is important in muscle regeneration and repair. Methods of using IGF-1 gene therapy techniques are described in U.S.

Application Serial No. 60/031,539 filed December 2, 1996, incorporated by reference herein in its entirety, including all tables, figures, and drawings. The characteristics of regeneration vary with the injury, but invariably involves proliferation of muscle precursor cells (MPC), fusion into myotubes, and reinnervation of the muscle. During muscle regeneration, IGF-I acts as a powerful stimulant of MPC proliferation and differentiation (Grounds, 1991, *Path. Res. Pract.* 187:1-22). Studies indicate that IGF-I is produced in satellite cells and nerves within 24 hours following muscle injury and remains elevated for several weeks. In regenerating rodent muscle, the pattern of IGF-I mRNA in damaged muscle parallels muscle precursor replication from the onset (18-24 hr) to the peak (5 days).

Reactive nerve sprouting is a wide-spread phenomenon in the nervous system. Nerve sprouting is believed to be initiated by locally activating factors. Intramuscular nerve sprouting can be detected about 4 days after muscle inactivation by crush denervation. Recent studies of Caroni and Schneider, 1994, *J. Neurosci.* 14:3378-3388, indicate that IGF-I is required for the induction of nerve sprouting. Studies also suggest that overexpression of IGF-I *in vivo* may by sufficient to enhance nerve sprouting.

Gene therapy provides an advantage to treating urinary incontinence over the existing pharmacological methods since gene therapy enables specific targeting of a therapeutic agent to a tissue or tissues. The vectors of the invention can be expressed in specific tissues. These vectors are useful in facilitating enhanced

expression in tissues as well as in targeting expression with tissue specificity. These vectors can be used to treat diseases by gene therapy by restricting expression of a gene encoded on the vector to targeted tissues. Vectors containing such sequences are able to provide gene delivery and controlled expression of recombinant genes within tissues; such expression can be at certain levels that are useful for gene therapy and other applications. These vectors can also be used to create transgenic animals for research or livestock improvement.

The ability of the expression vector to provide enhanced product secretion as well as direct expression to specific tissues allows the expression of many types of genes within many types of tissues. The above vectors can be used in gene therapy where a vector encoding a therapeutic product is introduced into a tissue so that tissue will express the therapeutic product. For example, the above vectors may be used for treating muscle atrophy associated with neurological, muscular, or systemic disease or aging by causing tissues to express certain trophic factors. These advantages can directly treat urinary incontinence since the disorder is often directly caused by muscle degeneration.

Expression of such vectors having an IGF-I encoding sequence in the body of a vertebrate, *e.g.*, a human, can produce both direct and indirect effects. The IGF-I produces direct effects by the direct action of the IGF-I polypeptide. However, indirect effects may also be produced due to the effect of the IGF-I inducing or turning on the expression of other genes.

The following are specific examples of preferred embodiments of the present invention and are not intended to limit the invention. These examples demonstrate how the expression vector systems of the present invention can be used in construction of various cellular or animal models, and how genes can be regulated by sequences within such vectors. The description and utility of such vectors and related vectors is discussed herein and is amplified upon in Schwartz et al., U.S. Patent No. 5,298,422, entitled "Myogenic Vector Systems,", and co-pending application Schwartz et al., Application No. 08/472,809, entitled "Expression Vector Systems and Method of Use", which are hereby specifically incorporated by reference herein, including drawings.

Below are provided examples of specific regions of 5' UTR and 3' UTR and/or 3' NCR regions of myogenic genes that can be used to provide certain functionalities to an expression vector, and thus within a transformed cell or animal containing such a vector. Those in the art will recognize that specific portions of these regions can be identified as that containing the functional nucleic acid sequence providing the desirable property, and such regions can be readily defined using routine deletion or mutagenic techniques or their equivalent. Such regions include the promoter, enhancer and cis- and transacting elements of a regulatable system. As noted herein, such controlling segments of nucleic acid may be inserted at any location on the vector, although there may be preferable sites as described herein.

Isolation of Chicken Skeletal α-Actin Gene

The nucleic acid sequence of the skeletal α-actin gene has been characterized in chicken, rat, mouse and human. Fornwald et al, 1982, Nucl. Acids Res. 10:3861-3876; R. Zakut, 1982, Nature 298:857-859; French et al, 1990, Gene(Amst.) 88:173-180; Hu et al, 1986, Mol. Cell. Biol. 6:15-25; Minty et al, 1986, *Mol. Cell. Biol.* 6:2137-2148. The skeletal α-actin gene is a member of the actin multigene family, which, in vertebrates, is made up of three distinct classes of actin isoforms termed as "cytoplasmic", "smooth muscle", and "striated" on the basis of their cellular distribution and pattern of expression in adult tissues. The striated actins, α-cardiac and α-skeletal, are co-expressed specifically in cardiac myocytes and skeletal myofibers. Expression of the α-cardiac and α-skeletal actin genes is sequentially up-regulated in developing cardiac and skeletal muscle with the skeletal isoform predominating in adult skeletal muscle. (Vandekerckhove & Weber, 1984, *J. Mol. Biol.* 179:391-413; McHugh et al., 1991, *Dev. Biol.* 148:442-458; Hayward & Schwartz, 1986, *J. Cell Biol.* 102:1485-1493.) The chicken skeletal α-actin gene is the most highly expressed gene in adult chicken skeletal muscle comprising approximately 8% of the poly(A) RNA.

Numerous experiments *in vitro* and *in vivo* have established that the regulatory sequences which confer cell type restricted and developmentally regulated expression to the skeletal α-actin gene are primarily concentrated in the immediate 5' promoter region. (Bergsma et al., 1986, *Mol. Cell. Biol.* 6: 2462-2475; Taylor et al., 1988, *Genomics.* 3(4): 323-36; Petropoulos

et al., 1989, *Mol. Cell. Biol.* 9:3785-3792; Carson et al., 1995, *Am. J. Physiol.* 268:C918-24.)

These regulatory sequences are highly conserved in the promoter regions of all of the known vertebrate skeletal α-actin genes from aves to man. Regulatory sequences derived from the chicken skeletal α-actin gene were utilized in construction of the IGF-I expression cassette, though other embodiments can utilize other actin or α-skeletal actin genes.

The primary sequences of the skeletal α-actin genes of the various species were deduced from overlapping cDNA clones. To obtain full genes, the cDNA clones were used to screen genomic DNA. For example, the 25 Kb EcoRI fragment of chicken genomic DNA isolated from a lambda Charon 4A vector, contains the 6.2 Kb skeletal α-actin gene on a single Hind*III* site of pBR322 is shown in Figure 1. Chang et al., *Mol. Cell. Biol.* 4:2498-2508 (1984). Nuclear transcription runoffs were used to map the transcriptional domain of the skeletal α-actin gene. The chicken skeletal α-actin control sequences have also been characterized (Bergsma et al., 1986, *Mol. Cell. Biol.* 6:2462-2475). DNA probes which encompassed portions of the 5' noncoding, promoter coding, and the contiguous 3' noncoding regions were cloned into M13 vectors which provided sense and antisense probes. Nuclei isolated from fibroblasts, myoblasts and day 19 embryonic muscle cells were used in *in vitro* transcription assays to extend RNA transcripts with radioactive tagged nucleotides. Labeled RNA hybridized to dotted DNA probes showed that transcription terminates approximately 1 kb downstream of the skeletal

α-actin gene's poly A addition site. This is within a 800 bp Pvu*II* fragment between +2800 and +3600 nucleotides from the start of transcription.

The 3' UTR and/or 3' NCR can be isolated by restriction endonuclease digestion of the 6.2 Kb actin gene with blunt cutter NaeI, which cuts 30 bp upstream of the translation termination codon TAA. Hind*III* releases the 3' most portion of the actin gene from the vector pBR322 (Figure 2). The 3'UTR and 3'NCR were used to prepare DNA constructs. The skeletal α-actin promoter and DNA flanking sequences (at least 411 nucleotides from the mRNA cap site) and DNA sequences extending through the skeletal 5' noncoding leader, first intron and up to the initiation of translation ATG, converted to a Nco*I* cloning site at +196, was liberated from a M13 double stranded DNA by Xba*I* and Nco*I* digestion, Klenow filled in and then linked into the XbaI and blunt SmaI sites of pBluescript II KS. The NcoI site is regenerated by this cloning step.

For certain vectors described in Schwartz et al., Application No. 08/472,809, the 3'UTR and 3'NCR on the 2.3 kb NaeI/HindIII fragment were directionally cloned into a blunt EcoRV site and the adjacent HindIII site of the pBluescript II KS vector cassette. The EcoRV and NaeI sites are destroyed. The restored NcoI site was used to insert cDNA sequences encoding polypeptides. Another cloning vector was constructed by inserting the skeletal α-actin promoter from -411 to -11 adjacent to the 3'UTR and 3'NCR. This expression vector eliminates the first intron and the skeletal actin 5' leader sequence. These two vectors were used in preparing DNA constructs to test the efficacy of the 3'UTR and 3' NCR.

Results obtained using vectors having a skeletal α-actin/IGF-I/skeletal α-actin expression cassette are described below, illustrating the intracellular expression of IGF-I from vector constructs and certain results of such expression.

For the exemplary vectors of the present invention, sequences including the skeletal α-actin promoter and first intron were utilized in conjunction with a IFG-I coding sequence and a hGH 3' UTR/poly(A) signal. Further results are presented below showing effects of IFG-I expression and certain comparative results with skeletal α-actin/IGF-I/skeletal α-actin containing vectors.

Expression Vector Construction Containing Human IGF-I Gene

Constructions containing the skeletal α-actin promoter were linked to the human IGF-I cDNA (SEQ ID NO. 1) by standard recombinant DNA techniques as known in the art. Examples of a generalized expression vector structure utilizing skeletal α-actin 5' and 3' sequences is shown in Figure 2. Certain specific vector constructs with IGF-I are shown in Figure 3.

A first construction (SK202 SVa) was made so that the SV40 poly A addition site and the small t-intron were linked to the 3'UTR of the IGF-I cDNA. The SV40 sequences were added to increase the stability of nuclear IGF-I RNA transcripts. Since the SV40 t-intron might not be entirely suitable in the expression of IGF-I in muscle cells, five other vectors were made.

The SK733 NcoI vector contains approximately 411 nucleotides of the skeletal α-actin promoter, the natural cap site, 5' untranslated leader and the first intron. An NcoI site was engineered to create a unique insertion cloning site for the cassette containing the IGF-I cDNA, in which the initiation ATG was also converted to an NcoI site.

The SK733IGF-I construction utilizes its own poly A site. An NaeI/HindIII fragment which incorporated the skeletal α-actin 3' UTR, poly A addition site, and terminating sequences was linked to SK202, SK733 NcoI, IGF-I and to SK733IGF-I which the IGF-I poly A site was deleted and replaced by that of skeletal α-actin. In this way IGF-I RNA transcripts containing the skeletal α-actin 3' UTR are stabilized and accumulate in skeletal muscle cells. In addition, by providing contiguous 3' NCR, IGF-I is buffered against outside genomic sequences and is thus more protected from position effects, when integrated into the genome. In addition, by providing natural terminating sequences, the additional regulatory sequences that mark the transcriptional domain of skeletal α-actin prevent read through transcription, improve tissue specificity, developmental timing and transcriptional activity. Presence of 3'NCR sequence allows for a single copy of the integrated vector to produce 40-100% of the transcriptional activity of the endogenous sequences.

The SK733 IGF-ISK2 plasmid construct (pIG0100A) is disclosed in the Schwartz et al. application referenced above, Application No. 08/472,809. This plasmid has an ampicillin resistance backbone and encodes for IGF-I. The plasmid construct pIG0335 is similar to pIG0100A but

it contains a Kanamycin resistance backbone, and is also disclosed in Schwartz et al., Application No. 08/472,809.

The exemplary plasmid vector, pIG0552 was constructed using pIG0100A and pIG0335B and additional constructs (pIG0376A and pVC0289A). A schematic representation of pIG0552 is shown in Fig. 4. The pIG0552B expression plasmid contains a hIGF-I gene expression cassette (Fig. 5) in a plasmid backbone containing a kanamycin-resistance (KanR) gene. The hIGF-I gene expression cassette of pIG0552B contains: 1) a promoter derived from the chicken skeletal α-actin promoter and first intron, 2) the human Insulin-like Growth Factor I (hIGF-I) cDNA, and 3) a 3' UTR/poly(A) signal from the human Growth Hormone (hGH) 3' untranslated region (3' UTR). The plasmid backbone is derived from pBluescript KS+ (Stratagene) with 1) the substitution of a kanamycin-resistance gene *(neo)* and prokaryotic promoter (pNEO, Pharmacia) in place of the ampicillin-resistance gene *(bla)* and 2) the deletion of the fl origin of replication.

Thus, the expression cassette described above differs from the original pIG0100 expression system specifically in the 3' UTR (pIG0100 contains skeletal actin 3' UTR; pIG0552 contains hGH 3' UTR). The hGH 3' UTR was substituted for the skeletal actin 3' UTR because it results in increased delivery of recombinant protein from skeletal muscle to systemic circulation. This result has been observed in both transgenic animal and non-viral gene therapy paradigms (i.e., both integrated and episomal template).

The actual construction of pIG0552B primarily involved three starting plasmids, pIG0100A, pIG0376A and pVC0289A. The process is shown schematically in Figs. 6, 7 and 8.

The chicken skeletal α-actin promoter and first intron and hIGF-1 cDNA were obtained from plasmid pIG0100A (R. Schwartz, Baylor College of Medicine). The hGH 3' UTR was obtained from plasmid pIG0376A (R. Schwartz, Baylor College of Medicine). pIG0100A contains the chicken skeletal α-actin promoter and first intron, human hIGF-1 cDNA, and chicken skeletal α-actin 3' untranslated region and 3' flanking sequence in pBluescript KS+. pIG0376A contains the chicken skeletal α-actin promoter and first intron, hGH leader sequence, hIGF-I cDNA, and hGH 3' UTR in pBluescript KS+. As indicated above, the plasmid backbone, pVC0289A, includes the kanamycin-resistance gene, pUC origin of replication, and a multicloning site.

The construction scheme used to produce pIG0552B from pIG0100A, pIG0376A, and pVC0289A required the construction of several intermediate plasmids. The first step in the construction of pIG0552B involved the transfer of the gene expression cassettes from pIG0100A and pIG0376A into pVC0289A, to produce pIG0335B and pIG0336A, respectively. pIG0335B was made by ligating the 3472 base pair (bp) NotI/Acc65I fragment containing the chicken skeletal α-actin promoter and first intron, hIGF-I cDNA, and chicken skeletal α-actin 3' UTR from pIG0100A into the NotI/Acc65I sites of pVC0289A. pIG0336C was made by ligating the 1918 bp NotI/Acc65I fragment containing the chicken skeletal α-actin

promoter and first intron, hGH leader sequence, human IGF-I cDNA, and hGH 3' UTR from pIG0376C into the NotI/Acc65I sites of pVC0289A. pIG0526A was constructed by ligating the 1132 bp BamHI fragment containing the chicken skeletal α-actin promoter and first intron and the hIGF-I cDNA from pIG0335B to the 3397 bp BamHI fragment containing the hGH 3' UTR in kanR backbone and a fragment of chicken skeletal α-actin promoter. pIG0526A contains a duplicated portion of the chicken skeletal α-actin promoter. To delete the duplicated portion of the chicken skeletal α-actin promoter, pIG0526A was digested with StuI and the 4057 bp fragment containing the chicken skeletal α-actin promoter and first intron, hIGF-I cDNA, and hGH 3' UTR in the KanR backbone was religated, creating pIG0533A.

pIG0533A contains a human ALU repeat sequence downstream of the hGH 3' UTR. The human ALU repeat sequence in pIG0533A was deleted to create plasmid pIG0552B. The 395 bp EcoO1091 (blunt-ended with T4 DNA polymerase)/BspEI fragment containing the 3' portion of the hIGF-I cDNA and hGH 3'UTR excluding the ALU repeat from pIG0533A was ligated to the 3175 bp XhoI (blunt-ended)/BspI fragment containing the KanR backbone, chicken skeletal α-actin promoter and first intron, and 5' portion of the hIGF-I cDNA from pIG0533A to produce the final plasmid, pIG0552B. The deletion of the ALU repeat greatly reduces the frequency of integration of the vector into a human chromosome. However, both pIG0552 and pIG0533 were found to produce approximately the same amounts of secreted IGF-I.

The actual nucleotide sequence of plasmid pIG0552 was determined by standard methods. The expected nucleotide sequence was assembled electronically using Vector NT version 1.2 (InforMax, Inc., Gaithersburg, MD) from previously determined sub-sequences or retrieved from GenBank as follows: (1) the plasmid backbone which is a derivative of pBluescript (Stratagene) in which the *bla* (Amp^r) gene has been replaced with the *neo* (Kan^r) gene from transposon tn5 (nucleotides 1 - 2261); (2) skeletal α-actin promoter (nucleotides 2262 - 2688); (3) skeletal α-actin 5' untranslated region (UTR) and first intron (nucleotides 2689 - 2884); (4) human IGF-I coding sequence and a portion of the hIGF-I 3' UTR (nucleotides 2885 - 3392); (5) pBluescript multiple cloning site (MCS, nucleotides 3393 - 3409), and (6) human growth hormone 3' UTR (nucleotides 3410 - 3509) and 3' flanking sequence (nucleotides 3510 - 3600; GenBank accession #J03071, HUMGHCSA). The expected and actual nucleotide sequences for pIG0552 are shown aligned in Table I below.

The first base of the plasmid backbone sequence is arbitrarily designated nucleotide #1. Sequence identities between the aligned sequences are indicated by "|". Selected sequence elements are labeled and underlined for reference. Although only one strand for each sequence is depicted, over 47% (nucleotides 1884 - 3599) of pIG0552 was sequenced with multiple reads on both strands. Nucleotides 2268 through 3599 of pIG0552 were identical to the expected sequence. This region of the plasmid includes virtually all of the skeletal α-actin promoter and 5' UTR, the entire hIGF-I coding sequence (**bolded**), the hGH 3' UTR and flanking sequence.

This confirms that this plasmid encodes a protein whose primary amino acid sequence matches that of the native human IGF-I protein.

A total of 8 nucleotide differences (indicated by "*") in other regions of the plasmid were observed between the actual and expected sequences. There is a single nucleotide deletion at position 21 in the expected sequence. This position is one base downstream from a Kpn I restriction site that is the last site in what remains of the pBluescript MCS. There is a single nucleotide difference at position 915 in the expected sequence. This position is in a non-critical region of the bacterial origin of replication. Finally, there are 6 nucleotide differences between positions 2262 and 2268 in the expected sequence. These positions are located at the cloning junction between the pBluescript MCS and the 5' end of the skeletal α-actin promoter sequence. The differences in this non-critical region are most likely the result of cloning artifacts. There is no evidence that any of the observed differences affect the relevant biological properties of pIG0552.

Table I
Plasmid pIG0552 Sequence

Upper Sequence: expected sequence for pIG0552 (nucleotides 1 - 3600) (SEQ ID NO. 2)

Lower Sequence: actual sequence for pIG0552 (nucleotides 1 - 3599) (SEQ ID NO. 3)

```
       Xho I      Apa I Kpn I *
 1     TCGAGGGGGGCCCGGTACCCAGCTTTTGTTCCCTTTAGTGAGGGTTAAT
       |||||||||||||||||||| |||||||||||||||||||||||||||||
 1     TCGAGGGGGGCCCGGTACC-AGCTTTTGTTCCCTTTAGTGAGGGTTAAT

51     TTCGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTT
       ||||||||||||||||||||||||||||||||||||||||||||||||||
50     TTCGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTT
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101  ATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAA
     ||||||||||||||||||||||||||||||||||||||||||||||||||
100  ATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAA

151  GCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTC
     ||||||||||||||||||||||||||||||||||||||||||||||||||
150  GCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTC

201  ACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAA
     ||||||||||||||||||||||||||||||||||||||||||||||||||
200  ACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAA

251  TCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCT
     ||||||||||||||||||||||||||||||||||||||||||||||||||
250  TCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCT

301  TCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGT
     ||||||||||||||||||||||||||||||||||||||||||||||||||
300  TCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGT

351  ATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATA
     ||||||||||||||||||||||||||||||||||||||||||||||||||
350  ATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATA

401  ACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGT
     ||||||||||||||||||||||||||||||||||||||||||||||||||
400  ACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGT

451  AAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGA
     ||||||||||||||||||||||||||||||||||||||||||||||||||
450  AAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGA

501  GCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGAC
     ||||||||||||||||||||||||||||||||||||||||||||||||||
500  GCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGAC

551  TATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCT
     ||||||||||||||||||||||||||||||||||||||||||||||||||
550  TATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCT

601  GTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGG
     ||||||||||||||||||||||||||||||||||||||||||||||||||
600  GTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGG

651  AAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGT
     ||||||||||||||||||||||||||||||||||||||||||||||||||
650  AAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGT

701  AGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCC
     ||||||||||||||||||||||||||||||||||||||||||||||||||
700  AGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCC

751  GACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAG
     ||||||||||||||||||||||||||||||||||||||||||||||||||
750  GACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAG
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801   ACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAG
      ||||||||||||||||||||||||||||||||||||||||||||||||||
800   ACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAG

851   CGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTAC
      ||||||||||||||||||||||||||||||||||||||||||||||||||
850   CGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTAC

901   GGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGT
      |||||||||||||| |||||||||||||||||||||||||||||||||||
900   GGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGT

951   TACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCG
      ||||||||||||||||||||||||||||||||||||||||||||||||||
950   TACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGC AAACAAACCACCG

1001  CTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAA
      ||||||||||||||||||||||||||||||||||||||||||||||||||
1000  CTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAA

1051  AAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCA
      ||||||||||||||||||||||||||||||||||||||||||||||||||
1050  AAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCA

1101  GAAGAACTCGTCAAGAAGGCGATAGAAGGCGATGCGCTGCGAATCGGGAG
      ||||||||||||||||||||||||||||||||||||||||||||||||||
1100  GAAGAACTCGTCAAGAAGGCGATAGAAGGCGATGCGCTGCGAATCGGGAG

1151  CGGCGATACCGTAAAGCACGAGGAAGCGGTCAGCCCATTCGCCGCCAAGC
      ||||||||||||||||||||||||||||||||||||||||||||||||||
1150  CGGCGATACCGTAAAGCACGAGGAAGCGGTCAGCCCATTCGCCGCCAAGC

1201  TCTTCAGCAATATCACGGGTAGCCAACGCTATGTCCTGATAGCGGTCCGC
      ||||||||||||||||||||||||||||||||||||||||||||||||||
1200  TCTTCAGCAATATCACGGGTAGCCAACGCTATGTCCTGATAGCGGTCCGC

1251  CACACCCAGCCGGCCACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCA
      ||||||||||||||||||||||||||||||||||||||||||||||||||
1250  CACACCCAGCCGGCCACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCA

1301  CCATGATATTCGGCAAGCAGGCATCGCCATGGGTCACGACGAGATCCTCG
      ||||||||||||||||||||||||||||||||||||||||||||||||||
1300  CCATGATATTCGGCAAGCAGGCATCGCCATGGGTCACGACGAGATCCTCG

1351  CCGTCGGGCATGCCCGCCTTGAGCCTGGCGAACAGTTCGGCTGGCGCGAG
      ||||||||||||||||||||||||||||||||||||||||||||||||||
1350  CCGTCGGGCATGCGCGCCTTGAGCCTGGCGAACAGTTCGGCTGGCGCGAG

1401  CCCCTGATGCTCTTCGTCCAGATCATCCTGATCGACAAGACCGGCTTCCA
      ||||||||||||||||||||||||||||||||||||||||||||||||||
1400  CCCCTGATGCTCTTCGTCCAGATCATCCTGATCGACAAGACCGGCTTCCA

1451  TCCGAGTACGTGCTCGCTCGATGCGATGTTTCGCTTGGTGGTCGAATGGG
      ||||||||||||||||||||||||||||||||||||||||||||||||||
1450  TCCGAGTACGTGCTCGCTCGATGCGATGTTTCGCTTGGTGGTCGAATGGG
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1501  CAGGTAGCCGGATCAAGCGTATGCAGCCGCCGCATTGCATCAGCCATGAT
      ||||||||||||||||||||||||||||||||||||||||||||||||||
1500  CAGGTAGCCGGATCAAGCGTATGCAGCCGCCGCATTGCATCAGCCATGAT

1551  GGATACTTTCTCGGCAGGAGCAAGGTGAGATGACAGGAGATCCTGCCCCG
      ||||||||||||||||||||||||||||||||||||||||||||||||||
1550  GGATACTTTCTCGGCAGGAGCAAGGTGAGATGACAGGAGATCCTGCCCCG

1601  GCACTTCGCCCAATAGCAGCCAGTCCCTTCCCGCTTCAGTGACAACGTCG
      ||||||||||||||||||||||||||||||||||||||||||||||||||
1600  GCACTTCGCCCAATAGCAGCCAGTCCCTTCCCGCTTCAGTGACAACGTCG

1651  AGCACAGCTGCGCAAGGAACGCCCGTCGTGGCCAGCCACGATAGCCGCGC
      ||||||||||||||||||||||||||||||||||||||||||||||||||
1650  AGCACAGCTGCGCAAGGAACGCCCGTCGTGGCCAGCCACGATAGCCGCGC

1701  TGCCTCGTCCTGCAGTTCATTCAGGGCACCGGACAGGTCGGTCTTGACAA
      ||||||||||||||||||||||||||||||||||||||||||||||||||
1700  TGCCTCGTCCTGCAGTTCATTCAGGGCACCGGACAGGTCGGTCTTGACAA

1751  AAAGAACCGGGCGCCCCTGCGCTGACAGCCGGAACACGGCGGCATCAGAG
      ||||||||||||||||||||||||||||||||||||||||||||||||||
1750  AAAGAACCGGGCGCCCCTGCGCTGACAGCCGGAACACGGCGGCATCAGAG

1801  CAGCCGATTGTCTGTTGTGCCCAGTCATAGCCGAATAGCCTCTCCACCCA
      ||||||||||||||||||||||||||||||||||||||||||||||||||
1800  CAGCCGATTGTCTGTTGTGCCCAGTCATAGCCGAATAGCCTCTCCACCCA

1851  AGCGGCCGGAGAACCTGCGTGCAATCCATCTTGTTCAATCATGCGAAACG
      ||||||||||||||||||||||||||||||||||||||||||||||||||
1850  AGCGGCCGGAGAACCTGCGTGCAATCCATCTTGTTCAATCATGCGAAACG

1901  ATCCTCATCCTGTCTCTTGATCAGATCTTGATCCCCTGCGCCATCAGATC
      | ||||||||||||||||||||||||||||||||||||||||||||||||
1900  ATCCTCATCCTGTCTCTTGATCAGATCTTGATCCCCTGCGCCATCAGATC

1951  CTTGGCGGCAAGAAAGCCATCCAGTTTACTTTGCAGGGCTTCCCAACCTT
      ||||||||||||||||||||||||||||||||||||||||||||||||||
1950  CTTGGCGGCAAGAAAGCCATCCAGTTTACTTTGCAGGGCTTCCCAACCTT

2001  ACCAGAGGGCGCCCCAGCTGGCAATTCCGGTTCGCTTGCTGTCCATAAAA
      ||||||||||||||||||||||||||||||||||||||||||||||||||
2000  ACCAGAGGGCGCCCCAGCTGGCAATTCCGGTTCGCTTGCTGTCCATAAAA

2051  CCGCCCAGTCTAGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCG
      |||||||||||||||||||| |||||||||||||||||||||||||||||
2050  CCGCCCAGTCTAGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCG

2101  CTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTG
      ||||||||||||||||||||||||||||||||||||||||||||||||||
2100  CTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTG

2151  GGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTG
       ||||||||||||| ||||||||||||||||||||||||||||||||||||
2150  GGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTG
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                                                     Sac I    Sac II
2201   AATTGTAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGGTGGC
       ||||||||||||||||||||||||||||||||||||||||||||||||||
2200   AATTGTAATACGACTCACTATAGGGCGAATTGGAGCTCCACCGCGGTGGC

                  SK promoter ->
       Not I        ***** *
2251   GGCCGCTCTAGCTAGAGTCTGCCTGCCCCCTGCCTGGCACAGCCCGTACC
       ||||||||||    | ||||||||||||||||||||||||||||||||||
2250   GGCCGCTCTAGAGCTTGGCTGCCTGCCCCCTGCCTGGCACAGCCCGTACC
          Xba I

2301   TGGCCGCACGCTCCCTCACAGGTGAAGCTCGAAAACTCCGTCCCCGTAAG
       ||||||||||||||||||||||||||||||||||||||||||||||||||
2300   TGGCCGCACGCTCCCTCACAGGTGAAGCTCGAAAACTCCGTCCCCGTAAG

2351   GAGCCCCGCTGCCCCCCGAGGCCTCCTCCCTCACGCCTCGCTGCGCTCCC
       ||||||||||||||||||||||||||||||||||||||||||||||||||
2350   GAGCCCCGCTGCCCCCCGAGGCCTCCTCCCTCACGCCTCGCTGCGCTCCC

2401   GGCTCCCGCACGGCCCTGGGAGAGGCCCCCACCGCTTCGTCCTTAACGGG
       ||||||||||||||||||||||||||||||||||||||||||||||||||
2400   GGCTCCCGCACGGCCCTGGGAGAGGCCCCCACCGCTTCGTCCTTAACGGG

2451   CCCGGCGGTGCCGGGGGATTATTTCGGCCCCGGCCCCGGGGGGGCCCGGC
       ||||||||||||||||||||||||||||||||||||||||||||||||||
2450   CCCGGCGGTGCCGGGGGATTATTTCGGCCCCGGCCCCGGGGGGGCCCGGC

2501   AGACGCTCCTTATACGGCCCGGCCTCGCTCACCTGGGCCGCGGCCAGGAG
       ||||||||||||||||||||||||||||||||||||||||||||||||||
2500   AGACGCTCCTTATACGGCCCGGCCTCGCTCACCTGGGCCGCGGCCAGGAG

2551   CGCCTTCTTTGGGCAGCGCCGGGCCGGGGCCGCGCCGGGCCCGACACCCA
       ||||||||||||||||||||||||||||||||||||||||||||||||||
2550   CGCCTTCTTTGGGCAGCGCCGGGCCGGGGCCGCGCCGGGCCCGACACCCA

2601   AATATGGCGACGGCCGGGGCCGCATTCCTGGGGGCCGGGCGGTGCTCCCG
       ||||||||||||||||||||||||||||||||||||||||||||||||||
2600   AATATGGCGACGGCCGGGGCCGCATTCCTGGGGGCCGGGCGGTGCTCCCG

                 "TATA"                          -1 (5' UTR)
2651   CCCGCCTCGATAAAAGGCTCCGGGGCCGGCGGCGGCCCACGAGCTACCCG
       ||||||||||||||||||||||||||||||||||||||||||||||||||
2650   CCCGCCTCGATAAAAGGCTCCGGGGCCGGCGGCGGCCCACGAGCTACCCG

2701   GAGGAGCGGGAGGCGTCTCTGCCAGCGGCCCGACGCGCAGTCAGCACAGG
       ||||||||||||||||||||||||||||||||||||||||||||||||||
2700   GAGGAGCGGGAGGCGTCTCTGCCAGCGGCCCGACGCGCAGTCAGCACAGG

2751   TAGGTGGGCACCGCGCCGTGCCGTGCCGTGCCGTGCCGCCCGGCGCCCCT
       |||||||||||||||||||||| ||||||||||||||||||||||||||||
2750   TAGGTGGGCACCGCGCCGTGCCGTGCCGTGCCGTGCCGCCCGGCGCCCCT

2801   TCGCGGGGCCGTCGTGTGGGCCCTCCGTGGGCCCCGCCGTCACCCTGAGC
       ||||||||||||||||||||||||||||||||||||||||||| ||||
2800   TCGCGGGGCCGTCGTGTGGGCCCTCCGTGGGCCCCGCCGTCACCCTGAGC
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                                        Met (IGF-I CDS ->)
2851  CTCACGGCCCCGTGCCCCGCAGACAGCCAGCACCATGGGAAAAATCAGCA
      ||||||||||||||||||||||||||||||||||||||||||||||||||
2850  CTCACGGCCCCGTGCCCCGCAGACAGCCAGCACCATGGGAAAAATCAGCA

2901  GTCTTCCAACCCAATTATTTAAGTGCTGCTTTTGTGATTTCTTGAAGGTG
      ||||||||||||||||||||||||||||||||||||||||||||||||||
2900  GTCTTCCAACCCAATTATTTAAGTGCTGCTTTTGTGATTTCTTGAAGGTG

2951  AAGATGCACACCATGTCCTCCTCGCATCTCTTCTACCTGGCGCTGTGCCT
      ||||||||||||||||||||||||||||||||||||||||||||||||||
2950  AAGATGCACACCATGTCCTCCTCGCATCTCTTCTACCTGGCGCTGTGCCT

3001  GCTCACCTTCACCAGCTCTGCCACGGCTGGACCGGAGACGCTCTGCGGGG
      ||||||||||||||||||||||||||||||||||||||||||||||||||
3000  GCTCACCTTCACCAGCTCTGCCACGGCTGGACCGGAGACGCTCTGCGGGG

3051  CTGAGCTGGTGGATGCTCTTCAGTTCGTGTGTGGAGACAGGGGCTTTTAT
      ||||||||||||||||||||||||||||||||||||||||||||||||||
3050  CTGAGCTGGTGGATGCTCTTCAGTTCGTGTGTGGAGACAGGGGCTTTTAT

3101  TTCAACAAGCCCACAGGGTATGGCTCCAGCAGTCGGAGGGCGCCTCAGAC
      ||||||||||||||||||||||||||||||||||||||||||||||||||
3100  TTCAACAAGCCCACAGGGTATGGCTCCAGCAGTCGGAGGGCGCCTCAGAC

3151  AGGCATCGTGGATGAGTGCTGCTTCCGGAGCTGTGATCTAAGGAGGCTGG
      ||||||||||||||||||||||||||||||||||||||||||||||||||
3150  AGGCATCGTGGATGAGTGCTGCTTCCGGAGCTGTGATCTAAGGAGGCTGG

3201  AGATGTATTGCGCACCCCTCAAGCCTGCCAAGTCAGCTCGCTCTGTCCGT
      ||||||||||||||||||||||||||||||||||||||||||||||||||
3200  AGATGTATTGCGCACCCCTCAAGCCTGCCAAGTCAGCTCGCTCTGTCCGT

3251  GCCCAGCGCCACACCGACATGCCCAAGACCCAGAAGGAAGTACATTTGAA
      ||||||||||||||||||||||||||||||||||||||||||||||||||
3250  GCCCAGCGCCACACCGACATGCCCAAGACCCAGAAGGAAGTACATTTGAA

                                                 Ter
3301  GAACGCAAGTAGAGGGAGTGCAGGAAACAAGAACTACAGGATGTAGGAAG
      ||||||||||||||||||||||||||||||||||||||||||||||||||
3300  GAACGCAAGTAGAGGGAGTGCAGGAAACAAGAACTACAGGATGTAGGAAG

3351  ACCCTCCTGAGGAGTGAAGAGTGACATGCCACCGCAGGATCCCCCGGGCT
      ||||||||||||||||||||||||||||||||||||||||||||||||||
3350  ACCCTCCTGAGGAGTGAAGAGTGACATGCCACCGCAGGATCCCCCGGGCT

3401  GCAGGAATTGGGTGGCATCCCTGTGACCCCTCCCCAGTGCCTCTCCTGGC
      ||||||||||||||||||||||||||||||||||||||||||||||||||
3400  GCAGGAATTGGGTGGCATCCCTGTGACCCCTCCCCAGTGCCTCTCCTGGC

                                           Poly (A)signal
3451  CCTGGAAGTTGCCACTCCAGTGCCCACCAGCCTTGTCCTAATAAAATTAA
      ||||||||||||||||||||||||||||||||||||||||||||||||||
3450  CCTGGAAGTTGCCACTCCAGTGCCCACCAGCCTTGTCCTAATAAAATTAA
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3501   GTTGCATCATTTTGTCTGACTAGGTGTCCTTCTATAATATTATGGGGTGG
       ||||||||||||||||||||||||||||||||||||||||||||||||||
3500   GTTGCATCATTTTGTCTGACTAGGTGTCCTTCTATAATATTATGGGGTGG

3551   AGGGGGGTGGTATGGAGCAAGGGGCAAGTTGGGAAGACAACCTGTAGGGC
       ||||||||||||||||||||||||||||||||||||||||||||||||||
3550   AGGGGGGTGGTATGGAGCAAGGGGCAAGTTGGGAAGACAACCTGTAGGGC
```

As noted above, evaluation of the exact sequence of pIG0552 demonstrated that a small number of sequence changes had occurred as compared to the resulting sequence predicted based on the sequences of the sequence components utilized. It was found that these changes did not occur in critical sequences. The presence of such changes in highly functional vectors provides further confirmation that vectors can incorporate a variety of different sequences while utilizing the same major sequence elements. Thus, the sequence disclosed is only exemplary.

Instead of the natural sequence coding for IGF-I, it is advantageous to utilize synthetic sequences which encode IGF-I. Such synthetic sequences have alternate codon usage from the natural sequence, and thus have dramatically different nucleotide sequences from the natural sequence. In particular, synthetic sequences can be used which have codon usage at least partially optimized for expression in a human. The natural sequences do not have such optimal codon usage. Preferably, substantially all the codons are optimized.

Optimal codon usage in humans is indicated by codon usage frequencies for highly expressed human genes, as shown in Fig. 9. The codon usage chart is from the program "Human_High.cod" from the Wisconsin Sequence Analysis Package, Version 8.1, Genetics Computer Group, Madison, WI. The codons which are most frequently used in highly expressed human genes are presumptively the

optimal codons for expression in human host cells, and thus form the basis for constructing a synthetic coding sequence.

However, rather than a sequence having fully optimized codon usage, it may be desirable to utilize an IGF-I encoding sequence which has optimized codon usage except in areas where the same amino acid is too close together or abundant to make uniform codon usage optimal.

In addition, other synthetic sequences can be used which have substantial portions of the codon usage optimized, for example, with at least 50%, 70%, 80% or 90% optimized codons. Other particular synthetic sequences for IGF-I can be selected by reference to the codon usage chart in Fig. 9. A sequence is selected by choosing a codon for each of the amino acids of the polypeptide sequences. DNA molecules corresponding to each of the polypeptides can then by constructed by routine chemical synthesis methods. For example, shorter oligonucleotides can be synthesized, and then ligated in the appropriate relationships to construct the full-length coding sequences.

A particular preferred synthetic IGF-I coding sequence is provided in SEQ ID NO. 4.

Preparation and Purification of IGF-I Plasmid

A. Preparation of the master cell bank

Competant cells were transfected with the IGF-I plasmid pIG0552 described above. The cells utilized for tranformation were MAX Efficiency DH5α™ Competent Cells (GIBCO BRL/Life Technologies). The Certificate of

Analysis supplied with the cells shows that they exhibit a Lac$^-$ phenotype (conferred by *lac* operon deletion), are inhibited by nitrofurantoin (demostrates *rec*A1 genotype), and are sensitive to antibiotics commonly used for plasmid stability (ampicillin, kanamycin and tetracycline). The published genotype of *E. coli* DH5α is F$^-$ϕ80d*lac*ZΔM15 Δ(*lac*ZYA-argF)U169 *end*A1 *rec*A1 *hsd*R17($r_K^- m_K^+$) *deo*R *thi*-1 *sup*E44 λ$^-$*gyr*A96 *rel*A1.

Prior to the creation of the master cell bank (MCB), two lots of pIG0552 DNA were produced (pIG0552B.16S and pIG0552B.100). Due to the low plasmid yields and long fermentation time, clone selection was included in the development of the MCB.

To begin clone selection, three colonies were picked from a fresh transformation plate, and designated clones X, Y, and Z. These colonies were simultaneously streaked onto LB-Kan agar plates and inoculated into 50 ml LB-Kan liquid medium. As a control, 20 μl of pIG0552B was also inoculated into 50 mL LB-Kan; this was designated clone B. The agar plates were incubated overnight at 37°C, wrapped with parafilm and stored at 4°C. The liquid cultures were shaken for 17 hours at 37°C, 250 rpm and then placed in an ice bath.

Each of the liquid cultures were measured for plasmid yield. Specific yields (mg/gDCW) were 5.1, <1, 2.8, and 6.2 for clones B, X, Y, and Z, respectively. Ethanol precipitations were performed on each of the undiluted cell lysates.

Clone Z showed improved yields; therefore, five isolated colonies from the pIG0552Z agar plate were picked and inoculated into 500 mL of LB-Kan liquid medium in a baffled Fernbach flask with a foam and

cheesecloth stopper. The culture was incubated with shaking for 16 hours, 37°C, 300 rpm and then placed in an ice water bath for cooling. The optical density of the culture was 3.4. Sterile 50% glycerol was cooled on ice, and 120 mL was added to the culture. The culture remained on ice with stirring while approximately 1 mL was dispensed into pre-labeled cryovials. Vials were transferred to the -20°C freezer. The next day, the vials were transferred to -80°C and then to liquid nitrogen for long term storage the following week.

To test the yield of the MCB, one vial was thawed, and 50 μL was used to inoculate 50 mL of LB-Kan liquid medium. After growth for 16 hours at 37°C, 250 rpm, the culture was analyzed for plasmid yield. The specific yield (mg/g DCW) was 5.2, which is within the expected limits for cultures started from a vial rather than an agar plate.

B. Bulk Preparation

Bulk hIGF-I plasmid is produced using batch fermentation with *Escherichia coli* (*E. coli*, DH5-α) as the host organism. The fermentation and subsequent recovery process steps are described below. For the description below, the process is described taking the following as a basis: 1 liter broth, density (A_{600nm}) of 83, 39.9 g/L dry cell weight (DCW), and 5 mg/g DCW specific yield of crude plasmid, measured at prepurification. These are approximations; actual quantities will vary depending on the productivity of the fermentation.

1. Solutions used in the process

Buffers, Media and Solutions Notes:

1) All quantities without ranges are nominal only.
2) Quantities with ranges are limited to the range specified.
3) Many buffers containing Tris-HCl are prepared with Tris base, using HCl for pH adjustment.

Kanamycin Sulfate - 20 mg/mL Kanamycin sulfate dissolved in WFI, then 0.2 micron filtered and stored in a -20°C freezer.

Primary Seed media (LB) - Tryptone 10 g/L, yeast extract 5 g/L, sodium chloride 10 g/L, water.

Secondary Seed Medium - Soytone 15 g/L, yeast extract 15 g/L, sodium chloride 10 g/L, water.

Fermentation media (two parts) - (1) Sterilized portion (90%): glycerol 50 ml/liter, yeast extract 50 g/L, $MgSO_4 \cdot 7H_2O$ 4-6 g/L, $(NH_4)_2SO_4$ 6 g/L; (2) Filtered portion (10%): thiamine hydrochloride 0.15 g/L, vitamin solution (see below) 1000x 3mL/L, K_2HPO_4 6 g/liter, KH_2PO_4 3-5g/L, trace metals solution (see below) 1000x 1-2 mL/L, 0.4 mL/L antifoam and 0025 - 0.5 mg/Kanamycin. All concentrations indicated based on the total volume.

Trace metals solution 1000x - $FeCl_3 \cdot 6H_2O$ 100 g/L, in sterile wash for irrigation.

Vitamin solution 1000x - Riboflavin 0.42 g/L, pantothenic acid 5.40 g/L, niacin 6.1 g/L, pyridoxine 1.4 g/L, biotin 0.06 g/L, folic acid 0.04 g/L, sterile water for irrigation, wrap in aluminum foil and store at 2-8°C.

Sodium hydroxide - 4-5 M NaOH in deionized water, for adjustment of the fermentation pH.

Phosphoric acid - 20% v/v concentrated phosphoric acid in deionized water, for adjustment of the fermentation pH.

Wash/resuspension buffer - 50 mM Tris-HCl, 10 mM EDTA, pH 8.0

Lysis buffer - 200 mM NaOH, 1% sodium dodecyl sulfate (SDS)

Neutralization buffer - A ratio of 35:13:8.75 of 3 M KOAc (pH 5.5), 5 M NaCl, and 7.5 M NH_4OAc.

RNase (100x) stock solution - 5 mg/mL RNase A, 10mM Tris-HCl, 15mM NaCl.

Water for irrigation - WFI in bags, purchased from a qualified vendor

Q Conditioning buffer - 2 M NaCl, 20 mM Tris-HCl, pH 8.0 prepared with WFI

Q Equilibration buffer - 0.625 M NaCl, 10 mM Tris-HCl, 1mM EDTA, pH 8.0 prepared with WFI

Q Wash buffer - Same as Q equilibration

Q Elution buffer- 0.75 M NaCl, 10 mM Tris-HCl, 1mM EDTA, pH 8.0

Q Regeneration solutions (1, 2, and 3) - (1) Same as Q and DEAE conditioning buffer, (2) 1 M NaOH, and(3) 1 M Acetic acid

Alkaline hydrolysis solution - 0.1 N NaOH prepared with WFI

Alkaline hydrolysis neutralization solution - 0.1 N HCl prepared with WFI

DEAE Conditioning buffer - same as Q conditioning buffer

DEAE Equilibration buffer - 0.33 M NaCl, 20 mM Tris-HCl, pH 7.5 prepared with WFI to a conductivity of 33 1 mS/cm

DEAE Wash buffer - Same as DEAE equilibration

DEAE Elution buffer - 0.4 mM NaCl, 20 mM Tris-HCl, pH 7.5, conductivity 39± 1 mS/cm

DEAE Regeneration solutions (1, 2, and 3) - (1) Same as Q and DEAE conditioning buffer, (2) 0.1 M NaOH, and(3) 0.1 M HCl

HIC Conditioning solution - 3.0 M Ammonium sulfate, prepared with WFI

HIC Equilibration solution - 1.5 M Ammonium sulfate, prepared with WFI

HIC Wash solution - same as HIC equilibration solution

HIC Regeneration solutions (1, 2, and 3) - (1) WFI, (2) 70% (v/v) ethanol, (3) 0.1 N NaOH prepared with WFI
Note: WFI is used twice (see flow diagram)

UF/DF diafiltration solutions (1-2) - (1) 1M NaCl prepared with WFI, (2) WFI

Final product dilution - WFI

2. <u>Fermentation Process and Isolation</u>

Media Preparation. Media for the seed step is prepared in pre-sterilized Pyrex containers in approximately 2 liter quantities and steam sterilized. The antibiotic is then added after filtering with a presterilized 0.2 micron filter. This sterile seed media is stored at 4°C until needed. Fermentation media is prepared immediately before use. The basal media is sterilized *in situ* and 0.2 micron filter-sterilized antibiotic is added to the fermentor by aseptic transfer.

Fermentation Process. The process is started with a seed vial from the master cell bank (MCB).

The two-stage seed process begins by preparing the seed culture in a biological safety hood. 10-25 mL of sterile seed media is added aseptically to a

presterilized flask. Filter-sterilized antibiotic solution is added to the appropriate final concentration (20-100 μg/mL). The culture is then inoculated with ≥200 μL of bacterial culture from the MCB vial. The flask is covered and placed in an incubator shaker. The seed culture is incubated at 37°C with shaking at about 250 rpm for two to six hours to develop the inoculum for the next stage. The next seed stage has the same antibiotic concentration, a volume of 1-10% of that used for the fermentation step, and is incubated similarly for two to eight hours.

The fermentation media contains 25-100 μg/mL of filter-sterilized antibiotic, which is added aseptically after media sterilization, to select against plasmid loss.

Fermentation starts when the inoculum from the seed is aseptically transferred to the fermentor. The fermentation is supplemented with up to 100 μg/mL antibiotic during the process to maintain selective pressure as cell density increases. Fermentation continues until an increase in dissolved oxygen indicates nutrient depletion, at which time the agitation is decreased and the culture cooled. After the temperature decreases to below 15°C, isolation steps are initiated. After fermentation, a sample of the culture is taken for a crude plasmid yield analysis, which is used to prepare for purification steps later in the process.

Isolation. The fermentation culture is centrifuged. The bacterial cell pellet is scraped from the centrifuge bowl(s) and transferred to presterilized 450 mL polypropylene bottles or resealable polyethylene

bags for resuspension and mixing. The cells are washed once with an equal volume of wash buffer, centrifuged again and either stored at 2° to 8°C for no longer than 24 hours or stored at -20°C until the time of use.

D. Purification

Prepurification. The cells are gently resuspended with the same buffer used for washing cells with a quantity sufficient for a total volume of about 7-12 mL/g of wet cell weight (WCW). Resuspended cells are gently transferred to a larger bottle or vessel and about 7-12 mL of lysis solution are added per gram (WCW) of starting cells to rupture cells and to denature cellular protein and chromosomal DNA. After addition of the lysis solution, the contents are gently mixed and held at room temperature (20-25°C) for about five minutes. Ice-cold neutralization solution is added, about 11-19 mL/g WCW, reducing the pH and precipitating cellular nucleic acids, protein, and chaotropic agent from the lysis buffer. The resulting suspension is held while cooling for a minimum of 1/2 hour.

Buffers and solutions prepared for this and other steps are 0.2 micron filtered and stored either in pre-sterilized pyrex bottles or in sterile and endotoxin-free disposable bags. The water used is sterile water for irrigation (WFI).

Solid-liquid separation is performed initially via centrifugation. Centrifugation is performed at 0-8°C. The supernatant, containing fine colloidal particles, is 0.3 micron-filtered to remove the remaining precipitate, completing the solid-liquid separation. The final container used is presterilized, washed again with 0.5N

sodium hydroxide and then triple-rinsed with WFI. The same treatment is applied to all product containers and transfer tubing used after this point with the exception of the containers used for pure bulk storage.

RNaseA stock solution, 100x concentration is stored at -20°C. RNA is digested after equilibrating the filtered alkaline lysis pool to room temperature and adding 0.01 v/v of RNase stock solution. The solution is incubated for a minimum of sixty minutes at a temperature of 30-45°C. The resulting solution is processed by chromatography immediately, or held overnight at 2 to 8°C in sterilized Pyrex containers.

Purification. The material is filtered through a 0.2 micron filter prior to chromatography. The supernatant containing plasmid, other cellular nucleic acids and protein is diluted three-fold with two volumes of WFI.

In the Q anion exchange step, the resin (Pharmacia Q high performance) is treated with 1 N NaOH for 30-35 minutes in the column as a precautionary measure. The column is conditioned with about 5 column volumes (CV) of Q and DEAE conditioning buffer, then equilibrated with about 5 CV of Q equilibration buffer (volume may be less if determined to be acceptable by pH and conductivity). The column feed rate is a linear velocity of about 155 cm/hr for all steps. The diluted feed is loaded to a maximum of one mg of crude plasmid per mL of resin. After the load, the column is washed with Q wash buffer for one additional CV after the column detector indicates output has leveled close to the baseline. The product is eluted with about 5 CV of elution buffer, with the actual peak on the chromatogram

indicating when eluate collection starts and ends. The column is regenerated with about 5 CV each of the three Q regeneration solutions. The resin is stored in the column until the next use after pumping 5-10 CV of 0.01 N NaOH through the column, or cycled again. The Q eluate may be stored at 2 to 8°C.

The RNA is checked using a crude plasmid analysis. If the ratio of the front RNA-containing peak to the second products containing peak is above a pre-set limit, a contingency alkaline hydrolysis and neutralization procedure is performed. Then 0.1 N NaOH is slowly added to the Q eluate with gentle mixing to achieve a final pH of 11.2-11.3. The pH of a sample of the treated solution is measured and recorded. The solution is held for about ten minutes at 20-25°C. Afterwards, 0.1 N HCl is added by slow addition with gentle mixing to neutralize the solution; the grid pH (between 7.5-8.0) is measured and recorded.

The pool is diluted about two-fold with WFI to give an appropriate salt concentration for the second purification step. As a precautionary measure in the DEAE anion exchange step, the resin (Tosohaas DEAE 650S) is treated with 0.1 N NaOH for 30-35 minutes in the column. The column is conditioned with about 5 CV of Q and DEAE conditioning buffer, then equilibrated with about 5 CV volumes of DEAE equilibration buffer. The column flow rate is a linear velocity of about 155 cm/hr for all steps. The feed is loaded to a maximum of 0.7 mg of crude plasmid per mL of resin. After the load, the column is washed with DEAE wash buffer for one additional CV after the column detector indicates output has leveled close to baseline. The elution takes place

with about 5 CV of DEAE elution buffer with the beginning and end of peak collection determined by the chromatogram. The column is regenerated with about 5 CV each of three regeneration solutions. The resin is stored until the next use after pumping 5-10 CV of 0.01 N NaOH through the column or cycled again. The DEAE eluate is stored at 2 to 8°C.

The DEAE eluate is diluted two-fold with hydrophobic interaction chromatography (HIC)-conditioning solution. The HIC resin (Tosohaas Phenyl 650S) is treated with 0.1 N NaOH for 30-35 minutes in the column, as a precautionary measure. The column is equilibrated with about 5 CV HIC of equilibration buffer. The column feed rate is a linear velocity of about 75 cm/hr for all steps. The feed is loaded to a maximum of 0.5 mg of crude plasmid per mL of resin and the flow through is collected. After the load, the column is washed with one CV of HIC equilibration buffer after the detector indicates the chromatogram is close to baseline; the wash is collected with the flow-through. The column is regenerated with about 5 - 10 CV of each of the three regeneration solutions. The resin is cycled again or stored until the next use after pumping 5-10 CV of 20% (v/v) ethanol through the column. The HIC eluate is stored at 2-8°C.

Alternatively, the purification process can be as described in U.S. Patent Application 60/022,157.

Myogenic Cell Cultures

Primary chicken myoblast cultures from breast muscles of day 11 white leghorn chick embryos were developed according to the protocol described in the

art. Grichnik et al., *Nucleic Acids Research* 14:1683-1701 (1986). Enriched myoblasts were plated at a density of 2 X 10^5 cells per 60 mm collagenized tissue culture dish.

Myogenic mammalian C_2C_{12} and Sol 8 cells (1 X 10^5) were subcultured onto 60 mm dishes one day before transfection.

DNA Transfer

Tissue culture cells were transfected with plasmid DNA by the calcium phosphate precipitation-glycerol shock protocol as known in the art. Wigler et al., *Cell* 14:725-731 (1978). A total of 10 μg of DNA was used to transfect each 60 mm dish of tissue culture cells. Transfections were done in quadruplicate and with three different MVS-CAT-MLC plasmid preparations to control for variations in DNA quality and plating density of cells.

CAT Assay

After transfection two populations of cells, coinciding with replicating myoblasts and post-fusion myotubes were harvested, and assayed for CAT activity as described in the art. Gorman et al., *Molec. Cell. Biol.* 2:1044-1051 (1982). Cell pellets were lysed by repetitive freeze thaw cycles in 50 μl of 250 mM Tris-HCl ph 7.5. The production of acetylated [^{14}C] chloramphenicol (0.5 μCi per assay, 57.8 mCi/mMol) was assayed for 90 minutes at 37° C. Acetylated chloramphenicol was monitored by autoradiography following thin layer chromatography on silica gel plates. Separated acetylated chloramphenicol spots were

quantitated by scanning on a Betagen phosphoimager screen. Data was expressed as the percentage of converted [^{14}C] chloramphenicol per μg cell protein. Protein concentration of cell extracts was determined by the method of Bradford, *Anal. Biochem.* 72:254-258 (1976)) at each time point to ensure uniformity in the assays.

Splicing of IGF-I Constructs

As described above, the pIG0100 construct was cloned to include the chicken skeletal actin promoter and intron (including the 5' and 3' splice sites), the human IGF-I 48 amino acid signal peptide, the 70 amino acid mature protein and the E peptide. The RNA produced from this expression system does not use the actin 3' splice site, instead it splices to a site in the IGF-I signal peptide sequence. The splicing has been confirmed by sequencing of RT-PCR products. It is believed that the resulting polypeptide has a 25 amino acid signal sequence, a form which is naturally occurring in muscle and many other tissues. Adamo *et al.*, 1994, *Adv. Exp. Med. Biol.* 343:1-11.

The pIG0552 construct contains the same upstream sequences as the pIG0100 construct with the human grwoth hormone 3' UTR instead of the chicken skeletal α-actin 3' UTR. It is believed that the splicing of the pIG0552 product is the same as for the pIG0100 product. It has been confirmed by agarose gel analysis of RT-PCR products that the products from both constructs are the same size.

Activity of Expression Vector Constructs

To determine the efficacy of actin promoter/gene IGF-I hybrid genes in mouse myogenic cells the expression vector was studied using these genes in the background of mammalian C_2C_{12} myoblasts by making a population of stable transfected C_2C_{12} myoblasts. The altered IGF-I expression levels were directly evaluated in these stable myoblast cell lines. Each IGF-I construction shown in Figure 3 was co-transfected with the drug selectable vector EMSV-Hygromycin into mouse C_2C_{12} cells. After two weeks of selection, a population of stable myoblasts was selected. A population of C_2C_{12} myoblasts stably transfected only with EMSV-Hygromycin served as the controls. Visual inspection of the transfected myoblast revealed several insights into the role of IGF-I on muscle cell differentiation that would not be obvious in transgenic mice. In general all of the myogenic cell lines containing IGF-I genes caused myoblasts in growth media (10% fetal calf serum) to replicate more extensively than controls. Changing culture medium to 2% horse serum initiates the differentiation process. In the process, control C_2C_{12} myoblasts fuse to form multinucleated myotubes over a period of four days. At the same cell density per culture dish, myoblasts containing SK733IGF-I, SK202IGF-I-SK, SK733IGF-I-SK1 and SK733IGF-I-SK2 fused at least two-to-three days earlier than C_2C_{12} or EMSV-Hygromycin control myoblasts.

In order to study the steady state accumulation of IGF-I MRNA in C_2C_{12} myoblasts, equal amounts of total cellular RNA was isolated from stably transfected C_2C_{12} myoblasts grown in growth media ("G") or differentiation

media ("D"). The RNA was electrophoretically separated on denaturing agarose gels, transferred onto nylon filters and probed with uniformly ^{32}p labeled full length human IGF-I cDNA under standard hybridization techniques. The intensity of the autoradiographic signal on X-ray film provides a relative measure of mRNA accumulation, an overall index of combined transcriptional activity and mRNA stability of the expression vectors. The IGF-I mRNA in vector, SK202IGF-I-3'SVa did not accumulate in myotubes above myoblast levels. This is a typical expression activity. The SK733IGF-I vector contains the IGF-I 3'UTR. The IGF-I mRNA from this vector accumulated in myotubes but at levels substantially lower than SK202IGF-I-SK or SK733IGFI-SK2. These latter two vectors contain the skeletal actin 3'UTR and 3'NCR. Since, the primary difference in these vectors is the 3'UTR, the increased stabilization of the RNA transcripts due to the skeletal 3'UTR accounts for about a 100-fold difference in RNA content.

In a similar assay, IGF-I was also produced at high levels from pIG0552 in C_2C_{12} cells.

Measurement of Secreted Levels of IGF-I from IGF-I Gene Delivery by the Expression Vector

In order to measure the amount of IGF-I synthesized and secreted into the media, differentiated myotube cultures were grown in minimal media (DMEM and 0.05% bovine serum albumin, RIA grade). SK733IGF-I-SK2 is the most effective construction to express IGF-I in muscle cells. IGF-I was assayed by both radioimmunoassays of tissue culture media and by immunoperoxidase staining of

cells. Increased levels of IGF-I during the fusion of several muscle cultures was found. The comparison of levels from different expression vectors are shown in Table II. In control cultures, the level of IGF-I was in the range of 0.2-0.5 ng/ml. In comparison, vector SK733IGF-I-SK2 (pIG0100A or pIG0335) has levels of IGF-I at least one hundred times greater.

Table II
IGF-I Levels in Stably Transfected C_2C_{12} Myoblasts

Construction	IGF-I (ng/ml of media/4 days)
SK202IGF-I-3'SVa	4.4
SK733IGF-I	3.8
SK733IGF-I-SK2	79.0
Control C_2C_{12}	0.5

In a similar manner, immunoperoxidase staining of myogenic cultures revealed the increased production of immunological reactive IGF-I in stable transfected myoblasts but not in the control EMSV-Hygromycin transfected myoblasts or in perfusion C_2C_{12} cells. Antibodies against the A and D regions were used at dilutions of 1:1000. All of the transfected lines including SK202IGF-I were positively immunoperoxidase stained. Thus, it is clear that enhanced levels of IGF-I are being synthesized and exported from the stable myoblasts.

Insertion of Expression Vectors into Transgenic Mice

Transgenic mice carrying hIGF-I containing vectors were generated by standard oocyte injection (Brinster, et al, *Proc. Natl. Acad. Sci. USA* 82:4438-4442 (1958)) and bred to demonstrate stable transmission of trans-

genes to subsequent generations. Transgenics were identified by polymerase chain reaction or Southern genomic DNA blotting analysis from tail cut DNA. Transgenics were tested for muscle specific expression of the transferred IGF-I vector by RNA blotting of total RNA isolated from several tissues. Independent transgenic mouse lines 5484, 5496, 5832, 5834 were generated with SK202IGF-I-3'SVa, containing the SV40 3' intron and poly A addition sequence. Mice from these strains were found to have weak expression, primarily in heart tissue, but very low levels were found in skeletal muscle and non-myogenic tissues such as the kidney and brain. Independent transgenic mouse lines 3357, 3359 generated with SK733IGF-I-3'SK2 (pIG0100A or pIG0335). Mice from these strains were found to have elevated expression levels of IGF-I. These levels are comparable to the endogenous mouse α-actin gene activity. These levels from SK733IGF-I-3'SK2 (pIG0100A or pIG0335) show at least 100-1000 fold greater accumulation of IGF-I mRNA in comparison to the levels produced by the SK202IGF-I-3'SVa vector. The addition of the skeletal α-actin 3'UTR and 3' flanking region allowed for a preferential increase in IGF-I RNA in skeletal muscle rather than cardiac. Thus, the 3'UTR and 3' NCR of skeletal α-actin enhance muscle specific gene expression.

Mice from these strains demonstrated increased muscle mass and reduced percentages of body fat as compared to the parental types. The use of human IGF-I in the mouse demonstrates the cross-species applicability of this particular gene.

In addition, by providing contiguous 3' NCR, IGF-I is buffered against outside genomic sequences and is thus more protected from position effects, when integrated into the genome. Also, by providing natural terminating sequences, the additional regulatory sequences that mark the transcriptional domain of skeletal a-actin prevent read through transcription, improve tissue specificity, developmental timing and transcriptional activity. Presence of 3'NCR sequence allows for a single copy of the integrated vector to produce 40-50% of the transcriptional activity of the endogenous sequences.

Somatic Gene Transfer to Skeletal Muscle In Vivo

To demonstrate an effect of the IGF-I encoding vectors as used in *in vivo* gene therapy, vectors were injected into adult muscle for the express purpose of expression of a particular polypeptide. The growth hormone-deficient mouse strain, *little*, was used in these studies. Vector SK733IGF-I-SK2 (pIG0100A or pIG0335), or control vector SKSK, was pelleted by sedimentation, dried under vacuum and punctured into the quadricep muscle (20 μg/pellet - 3 pellets/muscle) of 2 sets of 6 *little* mice. The entire muscle from each animal that received an inoculation was removed 2 weeks following introduction of the DNA and assayed for IGF-I protein in the tissue. The amount of IGF-I in each tissue was assayed by using a radioisotopic assay. A slight yet significant ($p>0.05$) increase was observed in IGF-I expression (Table III) from 4.2 ng to 6.9 ng IGF-I/100 μg total protein of muscle lysate in mice with

vector only (no IGF-I) for mice with the vector SK733IGF1-3'SK.

TABLE III
IGF-I Levels in Tissues of IGF-I Vector-Injected *little* MICE

Mouse#	Strain	Plasmid	IGF-I(ng/100ug)
776	*little*	PSKSK	4.2
777	*little*	PSKSK	4.2
778	*little*	PSKSK	4.5
779	*little*	PSKSK	3.9
780	*little*	PSKSK	3.9
781	*little*	PSKSK	4.2
Average 4.15+0.21			
782	*little*	pSK733IGFSK	4.5
783	*little*	pSK733IGFSK	6.3
784	*little*	pSK733IGFSK	8.2
785	*little*	pSK733IGFSK	6.9
786	*little*	pSK733IGFSK	8.4
787	*little*	pSK733IGFSK	7.0
Average 6.88±1.08			

Intramuscular Injections of a IGF-I Myogenic Vector in Diabetic Rats.

The effect of intramuscular injections of a muscle-specific DNA vector carrying the human insulin-like growth factor-I ("IGF-I") on diabetes-induced alterations in body and muscle weights, plasma glucose levels and the mRNA level from the injected IGF-I vector was examined. An IGF-I expressing vector was chosen for this work since injections of recombinant IGF-I have been shown to have anabolic effects in a number of models of cachexia.

Diabetes was induced in male Sprague-Dawley rats (175200 g) with intravenous injections of streptozotocin (STZ; 55 mg/kg) dissolved in sodium citrate buffer (0.05 M, pH 4.5). Control non-diabetic animals were age, weight and sex matched and received equal volume injections of vehicle. Diabetes was confirmed by the

onset of hyperglycemia, glucosuria, and reduced rate of growth. Three days following STZ administration, non-fasted animals were anesthetized with pentobarbital (50 mg/kg) and blood samples were obtained by cardiac puncture. Blood was transferred to EDTA-containing tubes, centrifuged at 3000 x g for 15 min and stored at -70°C. The gastrocnemius was injected bilaterally following direct visualization of the muscle via a cutaneous incision. The right gastrocnemius muscle of individual rats was injected with either 0, 50, 200, or 800 μg of IGF-I vector in 200 μl of isotonic saline solution. The contralateral (left) gastrocnemius received 200 μl injections of isotonic saline. The IGF-I vector used in this series of experiments was Sk-733-IGF-I-Sk2 as described above. Six days following intramuscular injection of muscle-specific vector, the animals were deprived of food (12-16 hrs) followed by euthanization by decapitation. Blood was then collected and the entire gastrocnemius muscle was removed (dissection from tendon to tendon).

For the analysis of vector effects on body and muscle weight dosage groups were matched on pre-vector injection body weight and only diabetic animals were included in the analysis. The plasma glucose criteria for inclusion in the analysis was a non-fasting plasma glucose level greater than 300 mg/100 ml. Pre-vector injection body weights were matched by only including animals with body weights between 175-195 gm. For the analysis of vector effects on plasma glucose levels the groups were matched on pre-vector injection plasma glucose levels. Intramuscular injections of IGF-I vector result in increased body weight (Mean ± SD;

Vehicle Only = 181.37 ± 6.17; 50 μg = 193.43 ± 5.71; 200 μg = 186.6 ± 8.01; 800 μg = 191.14 ± 7.54). This body weight increase is statistically significant at the 50 and 800 μg, but not the 200 μg, dose level (a priori t-test: Control vs. 50 μg, t = 3.57, df = 12; Control vs. 200 μg, t = 1.17, df 10; Control vs. 800 μg, t = 2.29, df = 12).

In addition to increasing body weight IGF-I vector injections also increase the weight of the vector injected gastrocnemius (Mean ± SD; Vehicle Only = 1.00 ± 0.08; 50 μg 1.10 ± 0.07; 200 μg = 1.07 ± 0.03; 800 μg = 1.09 ± 0.05) This increase in vector injected gastrocnemius weight is statistically significant at the 50 and 800 μg, but not the 200 μg, dose level (a priori t-test: Control vs. 50 μg, t = 2.32, df = 12; Control vs. 200 μg, t = 1.75, df 10; Control vs. 800 μg, t = 2.32, df = 12). The weight of the contralateral gastrocnemius was also increased but this increase did not reach statistical significant (Mean ± SD; Vehicle Only = 1.00 ± 0.07; 50 μg = 1.07 ± 0.06; 200 μg = 1.05 ± 0.01; 800 μg = 1.08 ± 0.06; a priori t-test: Control vs. 50 μg, t = 1.72, df = 12; Control vs. 200 μg, t = 1.43, df 10; Control vs. 800 μg, t = 2.11, df = 12).

The level of expression of the injected IGF-I construct was assessed by determining the level of IGF-I specific mRNA. Whole cell RNA isolated from the injected and control, contralateral, gastrocnemius, was treated with DNAase and subjected to reverse transcription using oligo-dT as a primer in order to generate cDNA replicas of mRNA. The cDNA was than reacted with IGF-I specific primers in a polymerase

chain reaction to estimate the level of expression of mRNA in the original muscle sample. The bands corresponding to IGF-I-specific primer amplified products were detected. The data indicates that the IGF-I vector IGF-I construct is being expressed at significant levels in the injected muscle. The control muscle showed no expression of human IGF-I.

Relative to the Control group fasting plasma glucose levels in the 50 μg IGF-I vector dose group were significantly lower (Mean ± SD; Vehicle Only = 277.14 ± 113.65; 50 μg = 155.42 ± 37.54; 200 μg = 224.06 ± 89.21; 800 μg = 216.57 ± 100.55 mg/100 ml). (a priori t-test: Control vs. 50 μg, t = 3.23, df = 12; Control vs. 200 μg, t = 1.04, df 17; Control vs. 800 μg, t = 1.09, df = 16).

These findings indicate that intramuscular injections of IGF-I vector (SK-7331-IGF-I-SK2) reduce diabetic hyperglycemia and increase body and muscle weight suggesting that IGF-I expression levels are sufficient to trigger an anabolic effect. The finding that the vector injected, but not the contralateral, gastrocnemius significantly increases in weight suggests a difference in local IGF-I concentration in the two muscles.

<u>Effect of Substitution of the hGH 3' UTR for the skeletal actin 3' UTR in skeletal actin - IGF-I transgenes on circulating concenetrations of hIGF-I in transgenic mice</u>

Transgenic mice containing the skeletal actin-IGF-I transgenes described in Figure 15 were generated. Serum samples were obtained and assayed for hIGF-I. Results

(Table IV) clearly demonstrate that transgenes containing the hGH 3' UTR elicit increased concentraions of hIGF-I in circulation relative to transgenes containing the skeletal actin 3' UTR. Other variables such as the presence/abscence or origin of intron and the origin of the 5' UTR appear to have little or no effect.

Table IV
Human IGF-I concentrations in serum of mice carrying skeletal actin - IGF-I transgenes.

Transgene	Animal ID	hIGF-I (ng/ml)
SISII	2813	3.0
448ISK	8219	6.5
448ISK	8226	ND[1]
448ISK	8230	ND
SIGh	2950	292.9
SIGh	5196	30.3
GIG	2338	253.7
GIG	2360	94.5
Non-transgenic control		ND

[1]ND - Not detectable (assay sensitivity is approximately 1 ng/ml).

As is shown by the data in the table, the GH 3' UTR sequences result in greatly enhanced serum concentrations (*i.e.*, enhanced secretion) of the encoded polypeptide as compared to the use of 3' sequences, such as skeletal actin 3' UTR, which provide higher retention of the product in the tissue. Thus, selection of 3' UTR sequences having appropriate secretion or retention promoting properties provides the ability to control the localization of the encoded product.

Enhanced Vector Expression in Intact Muscle

Intact plasmid DNA in a sterile 20% sucrose solution (wt/vol) can be injected into mature avian or mammalian muscle. Following a single injection the vector DNA is stable for at least 30 days as a non-integrated extrachromosomal circular DNA in muscle nuclei and, is transcriptionally active. Wolf et al., *Science*, vol. 247, pp. 1465-1468 (1990). However, greater than 99% of the injected DNA is degraded in muscle under the Wolff protocol (Wolff, et al, *BioTechniques* 11:4374-485 (1991)). This protocol can be improved by increasing the uptake of plasmid DNA into muscle and reducing vector degradation. The procedure of the present invention can use expression vector DNA coated with the relevant transcriptional regulatory factors, the human serum response factor and other human associated nuclear proteins, such as histone, and transcription initiation factors to enhance uptake and stability. The regulatory proteins protect the DNA against muscle nucleases and facilitate the uptake of the protein coated DNA into myogenic nuclei.

The expression vector forms a protein/DNA complex by the sequence specific binding of the serum response factor with the inner core CCXXXXXXGG (where X can be either A or T; SEQ ID NO. 6) of the serum response element and by the addition of histone. The interaction with the inner core of the promoter facilitates myogenic cell type restricted expression of the skeletal α-actin gene. The serum response factor, transcription initiation factor, transregulatory factor and histones are added to the expression vector by an in vitro

binding reaction to form a reconstituted protein/DNA complex.

Coating the Expression Vector System

A specific formulation involves coating the vector with elements of the transcription initiation complex and histone. This formulation is used both to enhance delivery of the vector to the cell and to enhance expression of the vector within the cell.

The following protocol was used to bacterially express and purify human serum response factor (SRF). Plasmid pARSRF-Nde is a T7 polymerase vector (Studier, F.W. and Moffatt, *J. Mol. Biol.* 189:113-130 (1986)) which produced full-length SRF protein upon IPTG (isopropyl-B-D-thiogalactopyranoside) induction. (Manak et al., *Genes and Development* 4:955-967 (1990)). E. coli BL21 harboring the plasmid was grown at 37°C to an OD_{600} of 0.4 in TYP medium supplemented with ampicillin (50 μg/ml). Synthesis of SRF was then induced with 1mM IPTG for 2.0 hr, after which cells were spun down, washed once in TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 7.0) and resuspended in a 40X packed cell volume and dialyzed against (10 mM HEPES [N-2 hydroxyethylpiperzine-N-2-ethansulfonic acid, pH 7.4], 60 mM KCl, 1mM 2-mercaptoethanol 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride and 10% glycerol). Cells were disrupted on ice by sonication. The lysate was clarified by centrifugation (15,000 xg for 20 min.) and the high speed supernatant containing overexpressed SRF was stored at -80C. Partial purification of SRF was done as follows. A 10 ml amount of the lysate was applied to a 10 ml phosphocellulose column equilibrated

with column buffer (same as dialysis buffer as described above) and 0.05% Nonidet P-40. The flow through fractions were collected and applied to a 5-ml heparin agarose column. The column was washed with 0.35 M KCl and SRF was eluted with 0.5 M KCl. SRF was then dialyzed and stored at -80°C.

Approximately, a ratio by weight of 5 to 1 SRF protein to expression vector DNA was allowed to incubate together in a solution containing 10 mM. Tris-HCl (pH 8.0, 0.1 mM EDTA, 2mM dithiothreitol, 5% glycerol plus 100 mM KCl. The binding of SRF to the actin promoter has been verified by DNA binding assays and by nuclease footprint protection assays as shown in the art. Transcription initiation factors such as the TATA box protein (TBP) and other initiation factors such as TFIIB, E and F are eluted from purified HeLa cell nuclei by the protocol of Dignam et al., *Mol. Cell. Biol.* 10:582-598 (1983) with 0.42M KCl in the above dialysis buffer. Nuclear lysates containing transcription initiation factors are mixed together with the SRF-DNA plasmid at a ratio of 10 parts protein to one part SRF-DNA to help form a preinitiation complex which is dialyzed for 24 hours. Finally, a crude histone preparation which is stripped from HeLa nuclei in 6M urea, 2M NaCl is dialyzed against low salt dialysis buffer. The full complement of histone are slowly added to a final ratio of 1 to 1 (histone to the SRF-protein DNA complex) to form nucleosome particles over nonprotected DNA. The addition of histone will protect regions of DNA to a greater extent than naked DNA from cellular nucleases.

The nucleoprotein complex is then further formulated with a lipid base, nonaqueous base and/or liposomes for direct injection into muscle. Because of the abundance of specific transcription factors, which contain nuclear targeting sequences, expression vector DNA is readily delivered, and taken up into muscle nuclei.

The vector can also be prepared in a formulation with other DNA binding compounds. For example, the vector can be prepared with polyvinyl pyrrolidone (PVP). PVP is a synthetic polymer consisting of linear 1-vinyl-2-pyrrolidone groups. PVP is commercially available with various degrees of polymerization and molecular weights. Pharmaceutical grade PVP is marketed under the trade names Plasdone (International Specialty Products, ISP) and Kollidon (BASF). ISP describes the typical properties of Plasdone C-30 in its product literature. Plasdone C-30 has a weight average molecular weight of 50,000 g/mol.

PVP is found to interact with DNA by hydrogen bonding. PVP is also found to protect DNA *in vitro* from nuclease (DNase 1) degradation. Reporter genes (CMV-CAT or CMV-β-gal) were formulated in PVP solutions and injected into rat tibialis muscles after surgical exposure. The results showed that DNA formulated at 3 mg/mL in 5% PVP in 150 mM NaCl led to the highest enhancement of gene expression over DNA formulated in saline. The levels of gene expression using lower molecular weight PVP (Plasdone C-15) were approximately 2-fold lower than levels of gene expression using formulations made with Plasdone C-30. When rat tibialis muscles were injected with DNA formulated in either

saline or 5% PVP (Plasdone C-30), immunochemical staining for β-galactosidase revealed that the staining was more widely distributed in muscles treated with the formulated DNA. The staining also showed that the PVP formulation resulted in an increase in the number of cells expressing β-gal and that these cells were distributed over a larger area as compared to DNA injected in saline. It is suggested that the increased tissue dispersion of DNA using PVP formulations is due to a hyper-osmotic effect in the muscle. DNA (3 mg/mL) in 5% PVP (Plasdone C-30) in 150 mM NaCl exerts an osmotic pressure of 341 $\pm$ 1 mOsm/kg H_2O.

An exemplary formulation of the hIGF-I plasmid is a three-vial system, with product components to be mixed just prior to use. The product components are:

1. Human IGF-I plasmid in sterile water;
2. Lyophilized PVP (polyvinylpyrrolidone; Plasdone C-30, Povidone U.S.P.); chemical formula $(C_6H_9NO)_n$;
3. 115 mM sodium citrate buffer (pH 4) in 5% NaCl.

The expression vector can also be delivered as described below.

Administration

Administration as used herein refers to the route of introduction of a vector or carrier of DNA into the body. Administration can be directly to a target tissue or by targeted delivery to the target tissue after systemic administration. In particular, the present invention can be used for treating disease by administration of the vector to the body in order to establishing controlled expression of any specific nucleic acid sequence within tissues at certain levels that are useful for gene therapy.

The preferred means for administration of vector and use of formulations for delivery are described above. The preferred embodiment is by direct injection using needle injection or hypospray.

The route of administration of any selected vector construct will depend on the particular use for the expression vectors. In general, a specific formulation for each vector construct used will focus on vector uptake with regard to the particular targeted tissue, followed by demonstration of efficacy. Uptake studies will include uptake assays to evaluate cellular uptake of the vectors and expression of the tissue specific DNA of choice. Such assays will also determine the localization of the target DNA after uptake, and establishing the requirements for maintenance of steady-state concentrations of expressed protein. Efficacy and cytotoxicity can then be tested. Toxicity will not only include cell viability but also cell function.

Muscle cells have the unique ability to take up DNA from the extracellular space after simple injection of DNA particles as a solution, suspension, or colloid into the muscle. Expression of DNA by this method can be sustained for several months.

Delivery of formulated DNA vectors involves incorporating DNA into macromolecular complexes that undergo endocytosis by the target cell. Such complexes may include lipids, proteins, carbohydrates, synthetic organic compounds, or inorganic compounds. The characteristics of the complex formed with the vector (size, charge, surface characteristics, composition) determines the bioavailability of the vector within the body. Other elements of the formulation function as

ligand which interact with specific receptors on the surface or interior of the cell. Other elements of the formulation function to enhance entry into the cell, release from the endosome, and entry into the nucleus.

Delivery can also be through use of DNA transporters. DNA transporters refers to molecules which bind to DNA vectors and are capable of being taken up by epidermal cells. DNA transporters contain a molecular complex capable of noncovalently binding to DNA and efficiently transporting the DNA through the cell membrane. It is preferable that the transporter also transport the DNA through the nuclear membrane. See, e.g., the following applications all of which (including drawings) are hereby incorporated by reference herein: (1) Woo et al., U.S. Serial No. 07/855,389, entitled "A DNA Transporter System and Method of Use,, filed March 20, 1992, now abandoned; (2) Woo et al., PCT/US93/02725, International Publ. WO93/18759, entitled "A DNA Transporter System and method of Use", (designating the U.S. and other countries) filed March 19, 1993; (3) a continuation-in-part application by Woo et al., entitled "Nucleic Acid Transporter Systems and Methods of Use", filed December 14, 1993, U.S. Serial No. 08/167,641; (4) Szoka et al., U.S. Serial No. 07/913,669, entitled "Self-Assembling Polynucleotide Delivery System", filed July 14, 1992 and (5) Szoka et al., PCT/US93/03406, International Publ. WO93/19768 entitled "Self-Assembling Polynucleotide Delivery System", (designating the U.S. and other countries) filed April 5, 1993.

Transfer of genes directly into muscle has been very effective. Experiments show that administration by

direct injection of DNA into muscle cells results in expression of the gene in the area of injection. Injection of plasmids containing IGF-I results in expression of the gene for months at relatively constant levels. The injected DNA appears to persist in an unintegrated extrachromosomal state. This means of transfer is the preferred embodiment.

Another preferred method of delivery involves a DNA transporter system. The DNA transporter system consists of particles containing several elements that are independently and non-covalently bound to DNA. Each element consists of a ligand which recognizes specific receptors or other functional groups such as a protein complexed with a cationic group that binds to DNA. Examples of cations which may be used are spermine, spermine derivatives, histone, cationic peptides and/or polylysine. One element is capable of binding both to the DNA vector and to a cell surface receptor on the target cell. Examples of such elements are organic compounds which interact with the asialoglycoprotein receptor, the folate receptor, the mannose-6-phosphate receptor, or the carnitine receptor. A second element is capable of binding both to the DNA vector and to a receptor on the nuclear membrane. The nuclear ligand is capable of recognizing and transporting a transporter system through a nuclear membrane. An example of such ligand is the nuclear targeting sequence from SV40 large T antigen or histone. A third element is capable of binding to both the DNA vector and to elements which induce episomal lysis. Examples include inactivated virus particles such as adenovirus, peptides related to

influenza virus hemagglutinin, or the GALA peptide described in the Skoka patent cited above.

Administration may also involve lipids. The lipids may form liposomes which are hollow spherical vesicles composed of lipids arranged in unilamellar, bilamellar, or multilamellar fashion and an internal aqueous space for entrapping water soluble compounds, such as DNA, ranging in size from 0.05 to several microns in diameter. Lipids may be useful without forming liposomes. Specific examples include the use of cationic lipids and complexes containing DOPE which interact with DNA and with the membrane of the target cell to facilitate entry of DNA into the cell.

Gene delivery can also be performed by transplanting genetically engineered cells. For example, immature muscle cells called myoblasts may be used to carry genes into the muscle fibers. Myoblasts genetically engineered to express recombinant human growth hormone can secrete the growth hormone into the animal's blood. Secretion of the incorporated gene can be sustained over periods up to 3 months.

Myoblasts eventually differentiate and fuse to existing muscle tissue. Because the cell is incorporated into an existing structure, it is not just tolerated but nurtured. Myoblasts can easily be obtained by taking muscle tissue from an individual who needs gene therapy and the genetically engineered cells can also be easily put back with out causing damage to the patient's muscle. Similarly, keratinocytes may be used to deliver genes to tissues. Large numbers of keratinocytes can be generated by cultivation of a small biopsy. The cultures can be prepared as stratified

sheets and when grafted to humans, generate epidermis which continues to improve in histotypic quality over many years. The keratinocytes are genetically engineered while in culture by transfecting the keratinocytes with the appropriate vector. Although keratinocytes are separated from the circulation by the basement membrane dividing the epidermis from the dermis, human keratinocytes secrete into circulation the protein produced.

Delivery may also involve the use of viral vectors. For example, an adenoviral vector may be constructed by replacing the E1 region of the virus genome with the vector elements described in this invention including promoter, 5'UTR, 3'UTR and nucleic acid cassette and introducing this recombinant genome into 293 cells which will package this gene into an infectious virus particle. Virus from this cell may then be used to infect tissue *ex vivo* or *in vivo* to introduce the vector into tissues leading to expression of the gene in the nucleic acid cassette.

The chosen method of delivery should result in expression of the gene product encoded within the nucleic acid cassette at levels which exert an appropriate biological effect. The rate of expression will depend upon the disease, the pharmacokinetics of the vector and gene product, and the route of administration, but should be between 1-1000 mg/kg of body weight/day. This level is readily determinable by standard methods. It could be more or less depending on the optimal dosing. The duration of treatment will extend through the course of the disease symptoms, possibly continuously. The number of doses will depend

upon disease delivery vehicle and efficacy data from clinical trials.

Animal Safety/Toxicology Studies

A. Acute 7-day and Subchronic 28-day Toxicity Studies

Acute 7-day and subchronic 28-day toxicity studies were conducted in dogs in compliance with the Good Laboratory Practice (GLP) Regulations of the United States Food and Drug Administration (21 CFR Part 58). The test articles and vehicle used in these studies were manufactured under cGMP procedures. Dogs were used because the mature human IGF-I (hIGF-I) which is expressed by the plasmid is identical to canine IGF-I.

The objective of the 7-day acute study was to investigate the potential acute toxicity of hIGF-I plasmid following a single intravenous injection in the dog. Four groups of beagle dogs, each consisting of two males and two females, were injected intravenously with the test article, hIGF-I plasmid formulated in polyvinylpyrrolidone (PVP), at dosage levels of 0.1, 1.0, and 12.0 mg/kg. The highest dose level was selected based on the maximum solubility of the test article in the vehicle and the total volume allowed for injection in dogs. The low dose corresponds to the minimum effective dose in preclinical animal studies in rodents.

A control group received the vehicle (PVP) only at the highest dose used with the test article. Two additional recovery groups (two males and two females in each) treated with the highest dose of test article and

control animals were kept for another week. The dogs were sacrificed on day 8 after injection, and the recovery groups were sacrificed on day 15 after injection.

An intravenous route of administration was used to mimic a "worst-case" scenario of systemic exposure of the test article. Mortality checks were performed twice daily throughout the study, and detailed examinations for clinical signs were performed hourly for the first four hours after dosing and daily during the observation period. Body weights were measured twice weekly during the last week of acclimation and throughout the observation period. Laboratory investigations (hematology, clinical biochemistry and urinalysis) were performed during the pretreatment period and on samples collected on days 2, 7, and 14 for all surviving animals. A complete necropsy was conducted on all animals, and selected organs including muscles were weighed.

There were neither abnormal clinical signs nor effects on body weight, food consumption, hematology, clinical biochemistry or urinalysis parameters. In addition, there were no differences in organ weights or gross pathological findings related to hIGF-I plasmid. Clinical signs consistent with histamine release were observed in control and high dose animals. These signs lasted for approximately two hours and were consistent with previous reports of histamine release in response to PVP observed in dogs. Thus, the signs were attributed to the PVP present in the dose formulations and not to the hIGF-I plasmid.

The objective of the subchronic study was to investigate the potential toxicity of hIGF-I plasmid during weekly intramuscular injection to beagle dogs for four weeks, followed by a four week recovery period. The intramuscular route is the intended route of administration in humans. Dogs were injected intramuscularly once weekly for four weeks with 0.1, 1.0, and 6.0 mg/kg. Each group consisted of three dogs per sex. Additional recovery groups (two dogs/sex) at the highest dose and control animals were observed for an additional 28 days. Mortality checks were performed at least twice daily throughout the study, and examinations for clinical signs of ill-health or reaction to treatment were performed at least twice daily following initiation of treatment. Individual body weights were determined on the day of randomization and weekly during the treatment and recovery periods. Food consumption was measured daily during the treatment and recovery periods. Ophthalmoscopy was performed once prior to the start of treatment and again during the last week of treatment (Week 4) and the last week of the recovery period (Week 8). Cardiovascular studies (electrocardiograms and systolic blood pressure measurements) and laboratory investigations (hematology, clinical biochemistry and urinalysis) were performed once prior to the start of treatment and again during weeks 4 and 8. In addition, serum samples were obtained on the same occasions and stored for possible future analysis. A complete necropsy was conducted on all animals sacrificed at the end of the treatment period. Selected organs were weighed, and a complete list of tissues was retained and microscopically evaluated.

There were neither abnormal clinical signs nor effects on body weight, food consumption, blood pressure, electrocardiograms, hematology, clinical biochemistry, urinalysis parameters, or ocular changes which were considered related to hIGF-I plasmid. As in the acute study, clinical signs consistent with histamine release were observed in control and high dose animals following administration of the control or test article. In response to occasional (4 out of 16) severe reactions, epinephrine was administered intravenously to prevent mortality. As before, these reactions were attributed to the PVP present in the test article and not to hIGF-I plasmid.

A pilot exploratory study conducted in two dogs confirmed that the observed clinical signs were due to histamine release. The dogs were injected intramuscularly with the vehicle at the high dose level (6 mg/kg) that elicited the clinical signs of histamine release. One of the dogs was pretreated with an H_1 histamine receptor blocker, diphenhydramine hydrochloride (Benadryl^R, 1 mg/kg). Both dogs developed the clinical signs, and pretreatment with the histamine antagonist did not abrogate the signs. Blood samples were analyzed for histamine levels, and they were approximately 100-fold or more higher than pretreatment levels in both dogs. These results suggest that the dosage of histamine blocker was inadequate. We believe that the effects seen in dogs are species specific and are unique to dogs. Experience with PVP used as a blood expander has not shown similar clinical signs in humans. There were no differences in organ weights or gross or

histopathological findings which were considered to be related to hIGF-I plasmid.

Thus, administration of hIGF-I plasmid by weekly intramuscular injection for four weeks produced no evidence of toxicity at doses up to 6 mg/kg/occasion. Clinical signs consistent with histamine release observed in control and high dose animals were attributed to the polyvinylpyrrolidone present in the dose formulations and not to hIGF-I plasmid.

B. Assay of Canine Serum for Anti-hIGF-I and Anti-DNA Antibodies

Serum samples obtained from dogs treated with hIGF-I plasmid in the subchronic (28 day) toxicity study were assayed for the presence of antibodies to rhIGF-I and double stranded (ds) DNA. Dogs were injected intramuscular with hIGF-I plasmid at dosages of 0 (vehicle control), 0.1, 1.0 and 12.0 mg/kg every 7 days for a period of 28 days. Serum samples (see outline in Table V) were obtained prior to the initiation of dosing (pre-bleed), at the end of dosing (day 27), and after a 28 day recovery phase (day 55). A total of 76 samples were assayed.

Table V
Outlines of serum samples assayed for antibodies to hIGF-I and to DNA

Dosage	Pre-bleed		Day 27		Day 55	
sex	F	M	F	M	F	M
Vehicle control	5	5	5	5	3	3
0.1 mg/kg BW	3	3	3	3		
1.0 mg/kg BW	3	3	3	3		
12.0 mg/kg BW	5	5	5	5	3	3

[a]Numbers represent the number of serum samples that were collected and assayed.

Antibodies to rhIGF-I were assayed using standard ELISA procedures. Results indicated that serum samples from treated dogs contained no detectable antibodies to rhIGF-I. Antibodies to dsDNA were assayed using an ELISA kit (The Binding Site, Inc., Birmingham, U.K.) designed to quantitate antibodies to dsDNA in human serum and was modified to quantify antibodies in dog serum. The anti-human IgG HRP conjugate was replaced with rabbit anti-dog IgG HRP conjugate as the second antibody. Results indicated that no serum samples from treated dogs contained detectable antibodies to ds DNA.

Pharmacokinetics/Biodistribution Studies

A. Time-Course of Expression of rhIGF-I in Rat Skeletal Muscle

Expression of rhIGF-I in tibialis anterior muscles of rats was determined at time points following intramuscular injection of hIGF-I plasmid. Human IGF-I plasmid formulated in polyvinyl pyrrolidone was injected bilaterally into tibialis anterior muscles (150 μg DNA/muscle) of male Fisher 344 rats (approximately 125 g BW). Rats were randomly divided into two groups with animals in one group receiving every other day injections of the immunosuppressant cyclosporine A (5 mg/kg BW) into the gluteus muscle for the duration of the experiment. Rats from each group were sacrificed at 24 hours, 48 hours, 7 days, 14 days, and 28 days following injection of hIGF-I plasmid (n=5-6 rats/group/time point). Tibialis anterior muscles were

harvested at these times and analyzed for expression of rhIGF-I by immunoradiometric assay (Diagnostic Systems Laboratories, Inc., Webster, TX).

Reverse transcriptase PCR analysis of total RNA from injected muscles did not reveal expression of hIGF-I mRNA at 24 or 48 hours post-injection. Expression of hIGF-I mRNA was observed at days 7, 14, and 28. Results for intramuscular content of rhIGF-I at 7, 14, and 28 days post-injection show that intramuscular content of rhIGF-I was similar at approximately 1.5-2.5 ng/g muscle between days 7 and 14 post-injection and decreased to approximately 35 percent of day 7 values by 28 days post-injection. Treatment with cyclosporine A to suppress the immune response did not affect ($p>.10$) intramuscular rhIGF-I content nor were antibodies to hIGF-I detected in serum of rats not receiving cyclosporine A treatment at 28 days post-injection. Together, these results suggest that the decrease in intramuscular rhIGF-I was not due to a host immune response. Human IGF-I was not detected in serum samples from injected rats at any time point.

B. <u>Determination of the Pharmacokinetics and Tissue Distribution of hIGF-I Plasmid</u>

The objective of these studies was to determine the pharmacokinetics and tissue distribution of hIGF-I plasmid following administration. Like the canine IGF-I, the mature guinea pig IGF-I polypeptide is identical to human IGF-I, making the guinea pig a suitable species to study the pharmacokinetics and biodistribution of hIGF-I plasmid. Two groups of Hartley guinea pigs were each injected once with either a low dose (0.1 mg/kg) or

a high dose (1.5 mg/kg) of hIGF-I plasmid formulated in 5% PVP by intramuscular or intravenous injections (50 males and 50 females per route of administration).

Five animals per sex from each treatment group were sacrificed at the following time intervals: 30 minutes, 1 hour, 6 hours, 12 hours, 1 day, 2 days, 1 week, 4 weeks and 3 months. As a control, one group of 10 male and 10 female guinea pigs per route of administration received the vehicle at the same volume/weight ratio as the high dose-treated group.

Gonads, lymph nodes, liver, spleen, kidney, lungs, heart, brain, bone marrow, muscle, and blood were collected at each sacrifice point. The blood was stored at 5°C, and the tissues were frozen in liquid nitrogen and stored at -70°C. DNA from blood samples was analyzed for the presence of the human IGF-I plasmid using a sensitive polymerase chain reaction (PCR) assay. If the plasmid was detected in the blood, the selected tissues were presumed to be positive and were not analyzed. If the plasmid was not detected in the blood sample, the DNA from the tissue samples was amplified in duplicate. One sample of each duplicate was spiked with the test plasmid at a copy number near the limit of detection to demonstrate the absence of any polymerase chain reaction (PCR) inhibition. Samples from animals treated using the high dose intramuscular and the intravenous routes have not yet been analyzed. Any positive tissue can be analyzed for human IGF-I messenger RNA to determine gene expression.

PCR evaluation of the blood samples from animals sacrificed through the second day following test article dosing indicated the presence of the DNA plasmid. By

day 7 after dosing, the plasmid had disappeared from the blood. PCR analysis of the samples (gonads, kidneys, liver, heart, and muscle tissues) from animals from the animals sacrificed three months after intramuscular injection of a low dose of hIGF-I plasmid (0.1 mg/kg) indicated elimination of most of the test plasmid at the injection site and in the peripheral systemic locations.

No clinical signs of toxicity were observed in any animal of either sex during the course of the study. All animals survived to the scheduled sacrifices. The animals for which terminal body weights were recorded (animals sacrificed after day 1) gained weight from the time of dosing to the time of sacrifice. There were no apparent significant chemically-related effects on body weight. Gross examination of selected tissues at necropsy revealed no abnormal findings at any time point with one exception. Brown foci on all lobes of the lungs were observed in one low dose female; however, this finding was not thought to be related to treatment with the test article.

In general, the data indicated that the hIGF-I plasmid is eliminated after three months. Out of a total of fifty tissues analyzed from ten animals given intramuscular injections of the low dose plasmid, only three positive signals were noted: one ovary, one liver and one muscle. The signals were sporadic and did not appear to be tissue-specific. All negative control tissues gave negative results.

Therefore, based on the complete lack of test article-related mortality, clinical signs of toxicity, effects of body weight, or gross lesions at necropsy

three months following exposure, the test article (as administered) is not toxic at the doses tested.

Cell Transfection and Transformation

One aspect of the present invention includes cells transfected with the vectors described above. Once the cells are transfected, the transformed cells will express the protein or RNA encoded for by the nucleic acid cassette. Examples of proteins include, but are not limited to polypeptide, glycoprotein, lipoprotein, phosphoprotein, or nucleoprotein.

The nucleic acid cassette which contains the genetic material of interest is positionally and sequentially oriented within the vectors such that the nucleic acid in the cassette can be transcribed into RNA and, when necessary, be translated into proteins or polypeptides in the transformed cells.

A variety of proteins can be expressed by the sequence in the nucleic acid cassette in the transformed cells. Those proteins which can be expressed may be located in the cytoplasm, nucleus, membranes (including the plasmalemma, nuclear membrane, endoplasmic reticulum or other internal membrane compartments), in organelles (including the mitochondria, peroxisome, lysosome, endosome or other organelles), or secreted. Those proteins may function as intracellular or extracellular structural elements, ligand, hormones, neurotransmitter, growth regulating factors, differentiation factors, gene-expression regulating factors, DNA-associated proteins, enzymes, serum proteins, receptors, carriers for small molecular weight organic or inorganic compounds, drugs, immunomodulators, oncogenes, tumor

suppressor, toxins, tumor antigens, or antigens. These proteins may have a natural sequence or a mutated sequence to enhance, inhibit, regulate, or eliminate their biological activity. A specific example of a protein to be expressed is hIGF-I.

In addition, the nucleic acid cassette can code for RNA. The RNA may function as a template for translation, as an antisense inhibitor of gene expression, as a triple-strand forming inhibitor of gene expression, as an enzyme (ribozyme) or as a ligand recognizing specific structural determinants on cellular structures for the purpose of modifying their activity. Specific examples include RNA molecules to inhibit the expression or function of prostaglandin synthase, lipo-oxenganse, histocompatibilty antigens (class I or class II), cell adhesion molecules, nitrous oxide synthase, β_2 microglobulin, oncogenes, and growth factors.

The compounds which can be incorporated are only limited by the availability of the nucleic acid sequence for the protein or polypeptide to be incorporated. One skilled in the art will readily recognize that as more proteins and polypeptides become identified they can be integrated into the vector system of the present invention and expressed in animal or human tissue.

Transfection can be done either by *in vivo* or *ex vivo* techniques. For example, muscle cells can be propagated in culture, transfected with the transforming gene, and then transplanted into muscle tissue. Alternatively, the vectors can be administered to the cells by the methods discussed above.

Methods of Use

A. Treatment of Urinary Incontinence by Direct Injection of a Gene Therapeutic

A treatment that specifically addresses urinary incontinence is the direct injection of a gene therapeutic that enhances the neuronal innervation and the integrity of muscles of the urinary system. The gene therapeutic can enhance muscular integrity and innervation by expressing a neurotrophic factor or growth factor, such as IGF-1, in the tissue injected with the gene therapeutic.

The gene therapeutic can be injected directly into the musculature using a variety of techniques based on the delivery of polymeric injectables to patients afflicted with urinary incontinence. Appell, 1995, *Obstetrics and Gynecology* 7: 393-396. Using these techniques, a gene therapeutic can be injected either transurethrally or periurethrally.

The gene therapeutic, which is injected suburethrally, can be accomplished via a needle placed directly through a cystoscope, or via a spinal needle inserted percutaneously and through the wall of the urethra while observing the delivery directly by urethroscopy. Kageyama et al., 1994, *J. Urol. 152*: 1473-1475. It has been discovered that the cause of incontinence, the tissue condition at the injection site, and the plane of delivery of the therapeutic can affect the treatment results. Appell, 1994, *Urol. Clin. N. Am. 21*: 177-182.

The gene therapeutic, once injected into the appropriate muscle or muscles, expresses a growth factor

or neurotrophic factor. IGF-1, which has a dual role of both stimulating muscle integrity and neuronal innervation, is one of the better suited growth factors for treating urinary incontinence. Suitable dosages for the administration of the gene therapeutic range from a low dose of 0.1 mg gene therapeutic/kg weight of the patient to a high dose of 1.5 mg gene therapeutic/kg weight of the patient. The gene therapeutic, such as a hIGF-I plasmid described herein, is formulated in 5% PVP for injection.

The principles set forth in the following examples demonstrate the efficacy of IGF-1 treatment of compromised musculature and can also be applied to the treatment of urinary incontinence.

B. Treatment of Urinary Incontinence by Catheter Mediated Delivery of a Gene Therapeutic

Another treatment that specifically addresses urinary incontinence is one that delivers a gene therapeutic to the bladder via an urethral catheter. The gene therapeutic can permeate the walls of the bladder into the surrounding muscle tissue by virtue of liposome, transporter, and viral technology discussed herein. The gene therapeutic is formulated in a solution comprising 0.5% - 50% PVP, preferably about 5% PVP.

The gene therapeutic, once permeated through the bladder walls, can enhance the neuronal innervation and integrity of muscles of the urinary system. The gene therapeutic can enhance muscular integrity and innervation by expressing a neurotrophic factor or

growth factor, such as IGF-1, in the musculature surrounding the bladder.

C. Treatment with Growth Hormone

Growth hormone is normally produced and secreted from the anterior pituitary and promotes linear growth in prepuberty children. Growth hormone acts on the liver and other tissues to stimulate the production of insulin-like growth factor I. This factor is, in turn, responsible for the growth promoting effects of growth hormone. Further, this factor serves as an indicator of overall growth hormone secretion. Serum IGF-I concentration increases in response to endogenous and exogenous administered growth hormone. These concentrations are low in growth hormone deficiency.

Insulin-like growth factors are one of the key factors that potentiate muscle development and muscle growth. Myoblasts naturally secrete IGF-I/IGF-II as well as its cognate binding proteins during the onset of fusion. This process coincides with the appearance of muscle specific gene products. In terminally differentiated muscle, signals propagated from passive stretch induced hypertrophy induce the expression of IGF genes. Many of the actions of IGFs on muscle result from interactions with the IGF-I receptor.

The intramuscular injection of an expression vector containing the sequence for IGF-I (for example, pIG0552) can be used to treat growth disorders. Vectors are designed to control the expression of IGF-I in a range of 100-400 ng/ml. Since intramuscular expression of vectors leads to expression of the product encoded by the nucleic acid cassette for several months, this

method provides a long-term inexpensive way to increase systemic blood concentration of IGF-I in patients with growth hormone deficiency.

D. Effect of IGF-I Vector Expression on Disuse Atrophy

Hindlimb suspension is a common experimental procedure used to induce atrophy of the calf muscles. The effects of hindlimb suspension are similar to those induced by cast immobilization and prolonged exposure to zero gravity.

Mice (12/group) were injected into the gastrocnemius and tibialis anterior muscles with either IGF-I containing vector (IGF-I) or control plasmid (PLAS) at days 0 and 7 of the suspension phase. The vectors were formulated at 3 mg/ml in poly-vinyl pyrrolidone (PVP) solution and administered at doses of 25 µl (75 µg DNA) into the tibialis anterior muscle and 50 µl (150 µg DNA) into the gastrocnemius muscle. This corresponds to a dosage of approximately 1 µg DNA/mg wet muscle weight.

Contractile force (strength) measurements and muscle weights were taken 1-2 days after cessation of hindlimb suspension. Animal not subjected to hindlimb suspension (NORM) were included for comparison.

Results, shown in Table VI, indicate that hindlimb suspension elicited an approximately 20-25% loss of muscle mass and strength and that treatment with IGF-I vector formulation reduced these effects ($p<.10$).

Table VI
Mean Values for Selected Parameters

Treatment	BW(g)	Tibialis weight (mg)	Tibialis (% body weight)	Tw tension (g)	Tet tension (g)	Gastroc. weight (mg)	Gastroc. (% body weight)
PLAS	27.33	47.39	.174	21.55	69.14	123.69	.454
IGF-I	27.09	50.79	.187	24.00	77.88	128.96	.476
NORM	31.14	59.72	.193	22.19	81.00	163.01	.525

E. Effects of IGF-I Vector Expression Following Crush Denervation

Sciatic nerve crush is a commonly used and well characterized model for elucidating the processes involved in degeneration and regeneration of neuromuscular function following trauma (M. Jaweed, 1994, The Physiological Basis of Rehabilitation, Downey et al., eds., p.543-561. Crush injury to the sciatic nerve results in rapid degeneration of axons distal to the lesion, loss of nerve conduction, and atrophy in the denervated muscle(s).

Early events in nerve regeneration begin within hours after crush injury initiating an ordered series of regenerative processes leading to re-establishment of neuromuscular synapses after 14-21 days and resumption of normal neuromuscular transmission after approximately 6 weeks in rodents. As a result of denervation, approximately 40-50% atrophy of affected myofibers and a concomitant decrease in isometric contractile force are observed after 14 days with eventual recovery to 80-90% of normal. Recoupment of muscular mass to pre-injury states requires several months.

Previous studies in rodents have indicated that daily administration of rhIGF-I protein can enhance

recovery of neuromuscular function following sciatic nerve crush.

Mature ICR strain mice were subjected to either unilateral sham (SHAM) operation or sciatic nerve crush. IGF-I containing vector formulation (IGF-I) or control plasmid (PLAS) was injected into the tibialis anterior and gastrocnemius muscles of the operated limb. Mice in the respective groups were subsequently injected with either IGF-I formulation or control plasmid formulation every 7 days thereafter.

The vectors were formulated at 3 mg/ml in poly-vinyl pyrrolidone (PVP) solution and administered at doses of 25 µl (75 µg DNA) into the tibialis anterior muscle and 50 µl (150 µg DNA) into the gastrocnemius muscle. This corresponds to a dosage of approximately 1 µg DNA/mg wet muscle weight.

Analyses for contractile force, muscle weight, electromyographic (EMG) activity, and nerve conduction velocity (NCV) were conducted at 14 day intervals following nerve crush. Measurement of EMG activity and NCV were performed using a Dantec Neuromatic 2000 EMG/EP system.

Sciatic nerve crush elicited marked muscle atrophy along with loss of nerve conduction and EMG activity. No significant differences in these parameters were noted between hIGF-I plasmid-treated and control animals at two weeks post-crush. However, treatment with hIGF-I plasmid elicited a modest improvement in gastrocnemius mass at three weeks post-crush along with striking improvements in EMG activity and NCV beginning three weeks post-crush. These data suggest that the beneficial effects of hIGF-I plasmid are manifested

relatively early (i.e., prior to three weeks) in the regenerative process.

These results indicate that expression from the IGF-I containing vector formulation enhances recovery from sciatic nerve crush.

F. Treatment of Muscle Atrophy Due To Age

Growth hormone levels decline with increasing age. The levels in healthy men and women above age of 55 are approximately one third lower than the levels in men and women 18 to 33. This is associated with a decrease in the concentration of IGF-I. The decline in growth hormone and IGF-I production correlate with the decrease in muscle mass, termed senile muscle atrophy, and increase in adiposity that occur in healthy human subjects. Administering growth hormone three times a week to healthy 61 to 81 year old men who had serum levels below those of healthy younger men increased the serum IGF-I levels to within the range found in young healthy adults. This increased level led to increased muscle mass and strength and reduced body fat. The secretion of growth hormone is regulated by a stimulatory (growth hormone releasing hormone) and an inhibitory (somatostatin) hypothalamic hormone.

The convenient cloning sites in the expression vectors of the present invention are used to construct vectors containing human growth hormone cDNA sequence, the human growth hormone releasing hormone (GHRH), or IGF-I. This versatility is important since the GHRH, GH, and IGF-I, while having equivalent desired effects on muscle mass, may have different side effects or kinetics which will affect their efficacy. The

expression of the growth factor releasing hormone might be more advantageous than the expression of either IGF-I or the growth hormone vectors transcripts. Since GHRH is reduced in the elderly it appears to be responsible for the lack of GH secretion rather than the anterior pituitary capability of synthesizing growth hormone, thus the increased expression of GHRH from muscle would increase GHRH levels in the systemic blood system and can allow for the natural diurnal secretion pattern of GH from the anterior pituitary. In this way, GHRH could act as the natural secretogogue, allowing for elevated secretion or release of GH from the hypothalamus of the elderly.

Thus, the application of vector systems described herein to express insulin-like growth factors through the injection of the pIG0552 or related vectors, the SK 733 IGF-I Sk2 vector, vectors expressing HG, or GHRH into adult muscle of the elderly is a long-term inexpensive way to increase systemic blood concentration of IGF-I in the elderly.

Administration of the vectors can be intravenously, through direct injection into the muscle or by any one of the methods described above. Dosages will depend on the severity of the disease and the amount of dosage is readily determinable by standard methods. The duration of treatment will extend through the course of the disease symptoms which can be continuously.

G. Treatment of Human Muscle Atrophies Induced by Neurological Dysfunction

Insulin-like growth factors are also known neurotrophic agents which maintain neuronal muscular

synapses, neuron integrity, and neuronal cell life under neurodegenerative conditions, and positively affect nerve regeneration. Since the expression vector driven genes are relatively insensitive to the innervation state of the muscle, they provide a direct and rather broad application for remedying certain kinds of human muscle atrophies caused by spinal cord injuries and neuromuscular diseases caused by drugs, diabetes, Type I disease, Type II diabetes, genetic diseases such as CHACOT-marie-tooth disease or certain other diseases. Moreover, IGF-I secretion can induce neurite outgrowth. In this treatment, the product of the vector acts as a neurotrophic agent secreted from injected muscle and as a hypertrophic agent to maintain muscle integrity.

Administration of the vectors can be intravenously, through direct injection or by any one of the methods described above. Dosages will depend on the severity of the disease and the amount of dosage is readily determinable by standard methods. The duration of treatment will extend through the course of the disease symptoms which can be continuously.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned as well as those inherent therein. The vector systems along with the methods, procedures treatments and vaccinations described herein are presently representative of preferred embodiments are exemplary and not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within

the spirit of the invention or defined by this scope with the claims.

It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein within departing from the scope and spirit of the invention.

All patents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: Coleman, Michael

(ii) TITLE OF INVENTION: TREATMENT FOR URINARY INCONTINENCE USING GENE THERAPY TECHNIQUES

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Lyon & Lyon
(B) STREET: 633 West Fifth Street Suite 4700
(C) CITY: Los Angeles
(D) STATE: California
(E) COUNTRY: U.S.A.
(F) ZIP: 90071-2066

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb storage
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: IBM P.C. DOS 5.0
(D) SOFTWARE: FastSEQ for Windows 2.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Warburg, Richard J.
(B) REGISTRATION NUMBER: 32,327
(C) REFERENCE/DOCKET NUMBER: 224/045

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (213) 489-1600
(B) TELEFAX: (213) 955-0440
(C) TELEX: 67-3510

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 462 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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AAGGTGAAGA TGCACACCAT GTCCTCCTCG CATCTCTTCT ACCTGGCGCT GTGCCTGCTC 120
ACCTTCACCA GCTCTGCCAC GGCTGGACCG GAGACGCTCT GCGGGGCTGA GCTGGTGGAT 180
GCTCTTCAGT TCGTGTGTGG AGACAGGGGC TTTTATTTCA ACAAGCCCAC AGGGTATGGC 240
TCCAGCAGTC GGAGGGCGCC TCAGACAGGC ATCGTGGATG AGTGCTGCTT CCGGAGCTGT 300
GATCTAAGGA GGCTGGAGAT GTATTGCGCA CCCCTCAAGC CTGCCAAGTC AGCTCGCTCT 360
GTCCGTGCCC AGCGCCACAC CGACATGCCC AAGACCCAGA AGGAAGTACA TTTGAAGAAC 420
GCAAGTAGAG GGAGTGCAGG AAACAAGAAC TACAGGATGT AG 462

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH 3600 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TCGAGGGGGG GCCCGGTACC CAGCTTTTGT TCCCTTTAGT GAGGGTTAAT TTCGAGCTTG 60
GCGTAATCAT GGTCATAGCT GTTTCCTGTG TGAAATTGTT ATCCGCTCAC AATTCCACAC 120
AACATACGAG CCGGAAGCAT AAAGTGTAAA GCCTGGGGTG CCTAATGAGT GAGCTAACTC 180
ACATTAATTG CGTTGCGCTC ACTGCCCGCT TTCCAGTCGG GAAACCTGTC GTGCCAGCTG 240
CATTAATGAA TCGGCCAACG CGCGGGGAGA GGCGGTTTGC GTATTGGGCG CTCTTCCGCT 300
TCCTCGCTCA CTGACTCGCT GCGCTCGGTC GTTCGGCTGC GGCGAGCGGT ATCAGCTCAC 360
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AGGCTCCGCC CCCCTGACGA GCATCACAAA AATCGACGCT CAAGTCAGAG GTGGCGAAAC 540
CCGACAGGAC TATAAAGATA CCAGGCGTTT CCCCCTGGAA GCTCCCTCGT GCGCTCTCCT 600
GTTCCGACCC TGCCGCTTAC CGGATACCTG TCCGCCTTTC TCCCTTCGGG AAGCGTGGCG 660
CTTTCTCATA GCTCACGCTG TAGGTATCTC AGTTCGGTGT AGGTCGTTCG CTCCAAGCTG 720
GGCTGTGTGC ACGAACCCCC CGTTCAGCCC GACCGCTGCG CCTTATCCGG TAACTATCGT 780
CTTGAGTCCA ACCCGGTAAG ACACGACTTA TCGCCACTGG CAGCAGCCAC TGGTAACAGG 840
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960
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TCTACGGGGT CTGACGCTCA GAAGAACTCG TCAAGAAGGC GATAGAAGGC GATGCGCTGC
1140
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1200
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1260
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1320
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1380
AACAGTTCGG CTGGCGCGAG CCCCTGATGC TCTTCGTCCA GATCATCCTG ATCGACAAGA
1440
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1500
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1860
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1920
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1980
TTGCAGGGCT TCCCAACCTT ACCAGAGGGC GCCCCAGCTG GCAATTCCGG TTCGCTTGCT
2040
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2100
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2460
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GGCCTCGCTC ACCTGGGCCG CGGCCAGGAG CGCCTTCTTT GGGCAGCGCC GGGCCGGGGC
2580
CGCGCCGGGC CCGACACCCA AATATGGCGA CGGCCGGGGC CGCATTCCTG GGGGCCGGGC
2640

GGTGCTCCCG CCCGCCTCGA TAAAAGGCTC CGGGGCCGGC GGCGGCCCAC GAGCTACCCG 2700
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CCCTCCGTGG GCCCCGCCGT CACCCTGAGC CTCACGGCCC CGTGCCCCGC AGACAGCCAG 2880
CACCATGGGA AAAATCAGCA GTCTTCCAAC CCAATTATTT AAGTGCTGCT TTTGTGATTT 2940
CTTGAAGGTG AAGATGCACA CCATGTCCTC CTCGCATCTC TTCTACCTGG CGCTGTGCCT 3000
GCTCACCTTC ACCAGCTCTG CCACGGCTGG ACCGGAGACG CTCTGCGGGG CTGAGCTGGT 3060
GGATGCTCTT CAGTTCGTGT GTGGAGACAG GGGCTTTTAT TTCAACAAGC CCACAGGGTA 3120
TGGCTCCAGC AGTCGGAGGG CGCCTCAGAC AGGCATCGTG GATGAGTGCT GCTTCCGGAG 3180
CTGTGATCTA AGGAGGCTGG AGATGTATTG CGCACCCCTC AAGCCTGCCA AGTCAGCTCG 3240
CTCTGTCCGT GCCCAGCGCC ACACCGACAT GCCCAAGACC CAGAAGGAAG TACATTTGAA 3300
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CTGTGACCCC TCCCCAGTGC CTCTCCTGGC CCTGGAAGTT GCCACTCCAG TGCCCACCAG 3480
CCTTGTCCTA ATAAAATTAA GTTGCATCAT TTTGTCTGAC TAGGTGTCCT TCTATAATAT 3540
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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A)	LENGTH:	3599 base pairs
(B)	TYPE:	nucleic acid
(C)	STRANDEDNESS:	single
(D)	TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TCGAGGGGGG GCCCGGTACC AGCTTTTGTT CCCTTTAGTG AGGGTTAATT TCGAGCTTGG 60
CGTAATCATG GTCATAGCTG TTTCCTGTGT GAAATTGTTA TCCGCTCACA ATTCCACACA 120
ACATACGAGC CGGAAGCATA AAGTGTAAAG CCTGGGGTGC CTAATGAGTG AGCTAACTCA 180
CATTAATTGC GTTGCGCTCA CTGCCCGCTT TCCAGTCGGG AAACCTGTCG TGCCAGCTGC 240
ATTAATGAAT CGGCCAACGC GCGGGGAGAG GCGGTTTGCG TATTGGGCGC TCTTCCGCTT 300
CCTCGCTCAC TGACTCGCTG CGCTCGGTCG TTCGGCTGCG GCGAGCGGTA TCAGCTCACT 360
CAAAGGCGGT AATACGGTTA TCCACAGAAT CAGGGGATAA CGCAGGAAAG AACATGTGAG 420

CAAAAGGCCA GCAAAAGGCC AGGAACCGTA AAAAGGCCGC GTTGCTGGCG TTTTTCCATA 480
GGCTCCGCCC CCCTGACGAG CATCACAAAA ATCGACGCTC AAGTCAGAGG TGGCGAAACC 540
CGACAGGACT ATAAAGATAC CAGGCGTTTC CCCCTGGAAG CTCCCTCGTG CGCTCTCCTG 600
TTCCGACCCT GCCGCTTACC GGATACCTGT CCGCCTTTCT CCCTTCGGGA AGCGTGGCGC 660
TTTCTCATAG CTCACGCTGT AGGTATCTCA GTTCGGTGTA GGTCGTTCGC TCCAAGCTGG 720
GCTGTGTGCA CGAACCCCCC GTTCAGCCCG ACCGCTGCGC CTTATCCGGT AACTATCGTC 780
TTGAGTCCAA CCCGGTAAGA CACGACTTAT CGCCACTGGC AGCAGCCACT GGTAACAGGA 840
TTAGCAGAGC GAGGTATGTA GGCGGTGCTA CAGAGTTCTT GAAGTGGTGG CCTAACTACG 900
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AAAGAGTTGG TAGCTCTTGA TCCGGCAAAC AAACCACCGC TGGTAGCGGT GGTTTTTTTG 1020
TTTGCAAGCA GCAGATTACG CGCAGAAAAA AAGGATCTCA AGAAGATCCT TTGATCTTTT 1080
CTACGGGGTC TGACGCTCAG AAGAACTCGT CAAGAAGGCG ATAGAAGGCG ATGCGCTGCG 1140
AATCGGGAGC GGCGATACCG TAAAGCACGA GGAAGCGGTC AGCCCATTCG CCGCCAAGCT 1200
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CGGCTTCCAT CCGAGTACGT GCTCGCTCGA TGCGATGTTT CGCTTGGTGG TCGAATGGGC 1500
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AACCTGCGTG CAATCCATCT TGTTCAATCA TGCGAAACGA TCCTCATCCT GTCTCTTGAT 1920
CAGATCTTGA TCCCCTGCGC CATCAGATCC TTGGCGGCAA GAAAGCCATC CAGTTTACTT 1980
TGCAGGGCTT CCCAACCTTA CCAGAGGGCG CCCCAGCTGG CAATTCCGGT TCGCTTGCTG 2040
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2340
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2400
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2460
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2520
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2580
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2640
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2700
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2820
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2880
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2940
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3060
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3120
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3180
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3240
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3300
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3360
GAGTGAAGAG TGACATGCCA CCGCAGGATC CCCCGGGCTG CAGGAATTGG GTGGCATCCC
3420
TGTGACCCCT CCCCAGTGCC TCTCCTGGCC CTGGAAGTTG CCACTCCAGT GCCCACCAGC
3480
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ATGGGGTGGA GGGGGGTGGT ATGGAGCAAG GGGCAAGTTG GGAAGACAAC CTGTAGGGC
3599

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 462 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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180
GCCCTGCAGT TCGTGTGCGG CGACCGCGGC TTCTACTTCA ACAAGCCCAC CGGCTACGGC
240
AGCAGCAGCC GCCGCGCCCC CCAGACCGGC ATCGTGGACG AGTGCTGCTT CCGCAGCTGC
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420
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462
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(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 153 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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Met Gly Lys Ile Ser Ser Leu Pro Thr Gln Leu Phe Lys Cys Cys Phe
 1               5                  10                  15

Cys Asp Phe Leu Lys Val Lys Met His Thr Met Ser Ser Ser His Leu
            20                  25                  30

Phe Tyr Leu Ala Leu Cys Leu Leu Thr Phe Thr Ser Ser Ala Thr Ala
        35                  40                  45

Gly Pro Glu Thr Leu Cys Gly Ala Glu Leu Val Asp Ala Leu Gln Phe
    50                  55                  60

Val Cys Gly Asp Arg Gly Phe Tyr Phe Asn Lys Pro Thr Gly Tyr Gly
65                  70                  75                  80

Ser Ser Ser Arg Arg Ala Pro Gln Thr Gly Ile Val Asp Glu Cys Cys
                85                  90                  95

Phe Arg Ser Cys Asp Leu Arg Arg Leu Glu Met Tyr Cys Ala Pro Leu
            100                 105                 110

Lys Pro Ala Lys Ser Ala Arg Ser Val Arg Ala Gln Arg His Thr Asp
        115                 120                 125

Met Pro Lys Thr Gln Lys Glu Val His Leu Lys Asn Ala Ser Arg Gly
    130                 135                 140
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Ser Ala Gly Asn Lys Asn Tyr Arg Met
145                 150
```

CLAIMS

We claim:

1. A method of treating urinary incontinence in mammals comprising the step of delivering a nucleic acid vector for the expression of a growth factor or neurotrophic factor in a tissue or tissues.

2. The method of claim 1, where the vector is contained within a formulation comprising a solution having between 0.5% and 50% PVP.

3. The method of claim 2, where the solution includes about 5% PVP.

4. The method of claim 1, where the tissue is myogenic.

5. The method of claim 4, where the myogenic tissue is selected from the group consisting of urethral sphincter musculature, detrusor musculature, and pelvic floor musculature.

6. The method of claim 1, where delivery is accomplished by injecting the vector using a hypodermic needle or hypospray apparatus.

7. The method of claim 1, where the vector comprises:

(a) a nucleic acid cassette containing a nucleotide sequence encoding a growth factor or neurotrophic factor;

(b) a 5' flanking region including one or more sequences necessary for expression of the nucleic

acid cassette, where the sequences include a promoter element selected from the group consisting of skeletal muscle α-actin promoter, smooth muscle γ-actin promoter, and cytomegalovirus promoter;

(c) a linker connecting the 5' flanking region to a nucleic acid, where the linker has a position for inserting the nucleic acid cassette, and where the linker lacks the coding sequence of a gene with which it is naturally associated; and

(d) a 3' flanking region, including a 3'-UTR or a 3'NCR or both, where the 3' flanking region is 3' to the position for inserting the nucleic acid cassette, and where the 3' flanking region comprises a sequence from a growth hormone 3'-UTR.

8. The method of claim 7, where the growth factor or neurotrophic factor is selected from the group consisting of PDGF, EGF, FGF, NGF, BDNF, IL-15, NT-3, NT-4/5, NT-6, CNTF, LIF, and GDNF.

9. The method of claim 7, where the growth factor is IGF-1 or IGF-II.

10. The method of claim 9, where the IGF-1 is human IGF-1.

11. The method of claim 10, where the human IGF-I gene is a synthetic sequence.

12. The method of claim 10, where the nucleotide sequence encoding human IGF-I has the sequence of SEQ ID NO. 4.

13. The method of claim 7, where the skeletal muscle α-actin gene promoter or smooth muscle γ-actin gene promoter is isolated from a chicken.

14. The method of claim 7, where the promoter from the skeletal muscle α-actin or smooth muscle γ-actin gene is isolated from a human.

15. The method of claim 7, where the growth hormone 3'-UTR is from a human growth hormone gene.

16. The method of claim 7, where the ALU repeat or ALU repeat-like sequence is deleted from the 3'-UTR.

17. The method of claim 7, where the IGF-I gene is human IGF-I, the promoter from a skeletal α-actin gene is from a chicken, and the growth hormone 3'-UTR is from a human growth hormone gene.

18. The method of claim 7, where the 5' flanking region or the 3' flanking region or both regulates expression of the nucleic acid cassette predominately in a specific tissue or tissues.

19. The method of claim 7, where the 5' flanking region includes a promoter, a TATA box, a Cap site and a first intron and intron/exon boundary in an appropriate relationship for expression of the nucleic acid cassette.

20. The method of claim 18, where the 5' flanking region further comprises a 5' mRNA leader sequence inserted between the promoter and the nucleic acid

cassette.

21. The method of claim 7, where the vector further comprises an intron/5' UTR from a chicken skeletal α-actin gene.

22. The method of claim 7, where the vector further comprises an antibiotic resistance gene.

23. The method of claim 7, where the vector comprises a nucleotide sequence having the same sequence as plasmid pIG0552.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/02051

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 526 756 A (BARD INC C R) 10 February 1993 see column 9, line 52 - column 10, line 4 ---	1-23
Y	WO 93 09236 A (BAYLOR COLLEGE MEDICINE) 13 May 1993 cited in the application see page 22; example 8 see claims 1-66 ---	1-23

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 June 1998	06/07/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Seegert, K

2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BARALDI M. ET AL: "Urine Retention due to intra-spinal cord injection of colchicine in rats: improved recovery of bladder function by monosialoganglioside GM1 and nerve growth factor administration" FUNCT.NEUR. /ITALY), vol. 6, no. 3, 1991, pages 235-238, XP002068722 see abstract -----	1-23

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/02051

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0526756 A	10-02-1993	CA 2071137 A	11-01-1993
		DE 69219418 D	05-06-1997
		DE 69219418 T	13-11-1997
		ES 2100255 T	16-06-1997
		JP 5186329 A	27-07-1993
		US 5686425 A	11-11-1997
		US 5739113 A	14-04-1998
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